A reverse genetics approach to *Drosophila* learning and memory.

A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow.

by

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March 1994

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The research reported within this thesis

is my own work, except where otherwise stated

and has not been submitted for any other degree.

Stephen F. Goodwin

Dedicated to my dad.

CONTENTS

CS	Conditioned stimulus
cAMP	adenosine 3', 5'-cyclic monophosphate
cGMP	guanosine 3', 5'-cyclic monophosphate
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
T_m (T_ms)	melting temperature
PEG	polyethylene glycol
CREB	cAMP response element-binding protein
ESDN	European Symposium on *Drosophila* Neurogenetics

ABBREVIATIONS

Chemicals

ATP	adenosine triphosphate
BCIP, X-phosphate	5-bromo-4-chloro-3-indoyl-phosphate
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DNA	2' deoxyribonucleic acid
dATP	2' deoxyadenosine triphosphate
dCTP	2' deoxycytidine triphosphate
dGTP	2' deoxyguanosine triphosphate
dNTP	2' deoxy (nucleotide) triphosphate
dTTP	2' deoxythymidine triphosphate
dUTP	2' deoxyuridine triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EtBr	ethidium bromide
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
MOPS	3-morpholinopropanesulfonic acid
mRNA	messenger RNA
NaPPi	sodium pyrophosphate
NTB	4-nitrobluetetrazoliumchloride
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer RNA

Measurements

bp	base pair
Ci	Curies
cm	centimetres
cpm	counts per minute
^{o}C	degrees centigrade
g	grammes
g	centrifugal force equal to gravitational acceleration

hr	hours
kb	kilobase pairs (10^3 bp)
kDa	kilodalton (10^3 dalton)
l	litres
mg	milligrammes
min	minutes
ml	millilitres
M	Molar
mM	milliMolar
µM	micromolar
ng	nanogrammes
nm	nanometres
nmol	nanomoles
nt	nucleotides
OD	optical density
pH	acidity [$-\log_{10}$(Molar concentration of H^+ ions)]
pmol	picomoles
rpm	revolutions per minute
sec	seconds
U	units
UV	ultra violet light
µCi	microCuries
µg	microgrammes
µl	microlitres
V	volts
vol	volume
W	watts

Miscellaneous

ORF	open reading frame
UTR	untranslated region
DNase I	deoxyribonuclease I
Rnase A	ribonuclease A

ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge the patience and generosity of all my colleagues in the Genetics Department, especially the "Media ladies", notably Agnes and Mattie for loans, cigarettes and abuse. Their enthusiasm is greatly appreciated. I want to express my sincere appreciation to the past and current members of the lab: to my supervisor, Kim "Have you done the Northern yet?" Kaiser; to former graduate students Steve "let me explain" Russell, Chris "amphibian" MacKenzie and Joyce "Kim!" Moore; to our current postdocs, particularily John "Skippy" Sentry and Kevin "I'm so tired" O'Dell; to fellow PhD students, Audrey "Shrimp" Duncanson, Colin "Pixie" Milligan, Simon "All I ever wanted to be was a hairdresser" Tomlinson, Diane "MacBison" Harbison, Andy "Pandy" Mounsey, Alan "Seagull" Paterson, Yang "The man", Tony "Dork" Dornan, Margaret "Baws" Adam, Steve "Flakey" Archibald; and of course a big one to Peggy "Peggers" Ennis. Together everyone provided a stimulating, friendly working environment, tolerated my frustrations and gave me intellectual, emotional and financial support throughout my PhD.

I thank Dan Kalderon, Jamie Paterson and Kevin O'Hare for plasmids, probes and primers; Tim Tully and Maria del Vecchio for the learning and memory experience; Kevin for statistical help; John, Audrey and Kim for reading and correcting this thesis, and for countless helpful conversations.

A penultimate big thanks goes to my family and friends, especially Mike, for supporting me throughout my PhD.

Last but not least, words cannot express how much I thank you Audrey; "Fancy a stir-fry, sausage?"

This work was partially supported by a postgraduate studentship from the Department of Education of Northern Ireland and a grant from the Department of Health and Social Security.

SUMMARY

A reverse genetics approach termed "site-selected" transposon mutagenesis (SSM) has been developed in *Drosophila*, which allows the detection and isolation of a P-element transposon into or near a cloned gene of interest (Kaiser and Goodwin, 1990; Kaiser, 1990). This approach exploits the mutagenic properties of strains carrying multiple P-elements (Robertson, 1988) and the polymerase chain reaction (PCR; Saiki et al., 1988). In a model system, SSM has been used successfully to isolate three independent P-element insertions in the *singed* gene, a known 'hot-spot' for P-element insertion, and six independent P-element insertions in the *ductin* gene, encoding the 16 kDa proteolipid subunit of the vacuolar H^+-ATPase. Furthermore, SSM has been used to identify two lines (*RI* ^{7I5} and *RI* ^{11D4}) containing P-element insertions in the regulatory (RI) subunit of *Drosophila* cAMP-dependent protein kinase (PKA; Kalderon and Rubin, 1988). Both lines have a defective P-element insertion within a 26 bp region containing multiple transcriptional start sites. Both lines were homozygous viable, presenting no obvious phenotype. Although the two mutant lines (*RI* ^{7I5} and *RI* ^{11D4}) were generated independently, they both have insertions identical in size, insertion site, and orientation and I consider them to be functionally equivalent. Northern analysis of mRNA produced by these lines reveals that RI transcription is disrupted relative to wild-type.

A number of single-gene defects have been isolated that perturb associative and nonassociative learning processes in *Drosophila*. The two most studied mutants are *dunce* (*dnc*), which encodes the cAMP-specific phosphodiesterase II (Byers et al., 1981; Kiger et al., 1981; Chen et al., 1986; Davis and Dauwalder, 1991; Qiu et al., 1991) and *rutabaga* (*rut*), which encodes a Ca^{2+}/Calmodulin-activated adenylate cyclase (Byers et al., 1981; Kiger et al., 1981; Livingstone et al., 1984; Levin et al., 1992). These findings implicated the cAMP signal transduction pathway in the neuromodulatory mechanisms underlying *Drosophila* learning and memory.

In order to test the part played by PKA in learning and memory, both lines were isogenised with a high learning index wild-type strain and tested in a classical olfactory conditioning paradigm (Tully and Quinn, 1985). They displayed a significant initial learning decrement with respect to wild-type. Preliminary experiments in a nonassociative learning paradigm, habituation of the jump reflex to olfactory cues (McKenna et al., 1989), suggested that waning of the jump reflex was normal. However, spontaneous recovery was higher than normal wild-type controls. *In situ* hybridisation to sections of wild-type adult heads showed the RI gene to be expressed throughout the CNS, but to be prominent in the mushroom bodies, supporting the theory that mushroom bodies are the chief sites mediating olfactory learning and memory (Heisenberg, 1985). Although a previous study involving expression of a peptide inhibitor of PKA was the first direct role for PKA in *Drosophila* learning (Drain et al., 1991), mutational studies provide the only way to investigate the relative roles of different isoforms of the regulatory and catalytic subunits.

Chapter 1

Introduction

1.1 Introduction to the genetic approach to learning and memory

The fruit fly *Drosophila melanogaster* is an excellent model system for the genetic analysis of learning and memory. It has two major benefits. Firstly it is possible, due to unparalleled genetic sophistication, to systematically screen for all genes that can mutate to produce a given phenotype and secondly its ability to display different types of learning and memory, both associative and nonassociative behaviours (Aceves-Pina, et al., 1983; Dudai, 1988; Tully, et al., 1990; Tully and Quinn, 1985). Indeed several experimental paradigms have been developed which can assess the ability of flies to learn and remember and it has been possible to screen for mutations affecting these processes (Boynton and Tully, 1992; Quinn, et al., 1974; Tully and Quinn, 1985). It is assumed that the flie's ability to acquire, store and retrieve new information is encoded by specific genes with each gene product serving a specific function. Mutations in one of these genes could eliminate or alter the function of its product, which could in turn disrupt or alter the ability of a fly to learn and/or remember. Studying the structure, function and regulation of individual genes that participate in learning and memory could help elucidate the molecular mechanism(s) underlying these processes. Single gene behavioural and neurological mutants in *Drosophila* have already provided information on how the nervous system is assembled, and what components underlie the functions of the cells in the system. Indeed, these components may be common to lower and higher organisms.

1.2 The Behaviours

Fruit flies can exhibit several different types of learning and memory. These include two general types of learning, nonassociative and associative. Habituation (a decrement in behavioural response) and sensitisation (an increment in behavioural response) are forms of nonassociative learning that result from exposure only to one environmental stimulus. In contrast, associative learning results from the temporal association of two stimuli, one

of which acts as a natural "reinforcer" of the behavioural response. A variety of experimental paradigms, using a range of sensory modalities, have been developed that can assess the flies ability to learn and remember.

The first reliable measurement of associative learning in *Drosophila* was demonstrated by Quinn et al., 1974. The procedure was as follows: a group of about 40 flies were shaken into the end of a start tube (Figure 1.1). Induced by phototactic cues, the flies ran into a grid tube coated with one or other odour (A or B). The flies were exposed alternatively to two chemical odours, the first of which was paired to an electric shock (odour A). After this experience the flies were shaken back into the 'start tube'. The 'start tube' was then shifted to a new position where the flies experienced a different odour without electric shock (odour B). After three training cycles, the flies were tested by exposing them sequentially to the odours without shock. Flies avoiding the odour tubes were counted. Odour concentrations were chosen so that naive flies avoided each odour equally. A learning index was calculated as the fraction of flies avoiding the shock-associated odour (CS^+) minus the fraction of flies avoiding the control odour without shock (CS^-). A reciprocal experiment, in which A was the control and B the shock-associated odorant was then conducted on a different group of flies from the same stock, to control for odour bias and nonassociative conditioning. The final learning index was the average of the two reciprocal experiments. Thus for perfect learning, i.e. all the flies avoided the CS^+ and none avoided the CS^-, then the learning index would be 1.0. If the flies failed to learn, i.e. the flies would avoid the CS^+ and the CS^- equally, the learning index would be 0. The wild-type strain Canton S typically reached indices of 0.3. The conditioned avoidance persists for at least one hour and if training is repeated four times at 2 hour intervals, a memory trace is found even after 24 hours.

Tully (1991) has proposed two factors which may act to give less than optimal learning indices in the paradigm of Quinn et al., 1974. Firstly, flies which for some reason do not enter the training tube receive no proper reinforcement. Secondly, flies have to suppress phototactic cues and avoid the paired odour. To address these imperfections, Tully and Quinn (1985) developed a classical conditioning paradigm

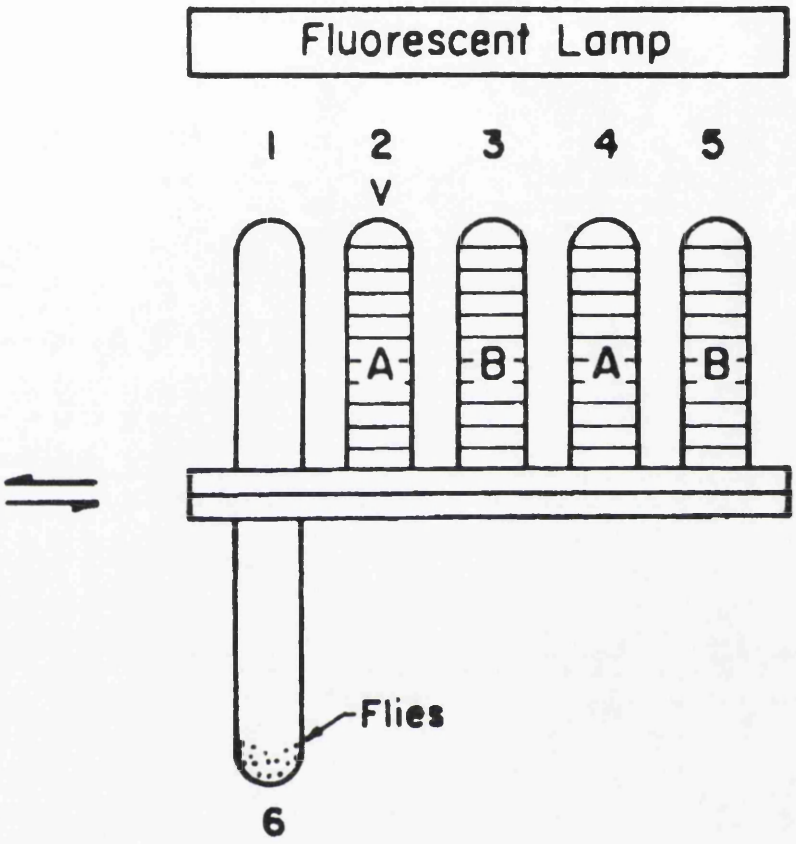


Figure 1.1. Shock avoidance conditioning apparatus. Tube 1 is the rest tube; 2 and 3 are for training; 4 and 5 are for testing. Tube 6 is the start tube. Horizontal stripes in tubes indicate grids. A and B, odourants 3-octanol and 4-methylcyclohexanol, respectively. V, voltage on the grid. (Taken from Quinn et al., 1974).

where all the flies receive an electric shock devoid of phototactic cues. Instead, flies were sequestered in the electrified odour containing chamber and subjected to a controlled series of electric shocks in the presence of controlled air currents carrying the appropriate odour. In Tully and Quinn's 'hands', 93% of wild-type flies avoid the shock-associated odour and only 4% avoid the control odour, yielding learning indices of about 0.9 (Figure 1.2). A detailed description of this paradigm is presented in section 5.1 of this thesis.

In another learning paradigm it has been demonstrated that flies can learn to associate olfactory cues coupled with a food reward (sugar) instead of electric shock (Tempel et al., 1983). Interestingly, it would appear that the processes of acquisition and memory are mechanistically different from those observed for classical conditioning using negative reinforcement (Tempel et al., 1983; Heisenberg, 1989). Memory using positive reinforcement is very long lived, typically decaying to half its initial value after 2.4 hours. Unlike negatively reinforced learning, positive reinforcement is extremely sensitive to 'extinction', being extinguished after only one presentation of the CS without reinforcer. Extinction, a property of classical conditioning, is a disappearance of memory when the odour is presented alone without the reward.

Visual cues have also been used instead of odours as conditioned stimuli in associative learning paradigms (Quinn et al., 1974; Spatz et al., 1974; Menne and Spatz, 1977). Again like reward learning, there are mechanistic differences in the acquisition and retention of memory.

Aceves-Pina and Quinn, (1979) demonstrated that behavioural conditioning is not restricted to adult flies. Larvae can learn to associate olfactory cues and negative reinforcement. In this classical conditioning paradigm a memory trace can still be detected in the adult fly more than a week later (Tully and Kruse, unpublished, cited in Tully, 1991). Manning, (1967) has shown that the memory of odour habituation is retained through metamorphosis.

Flies can also exhibit nonassociative behaviours (Duerr and Quinn, 1982; Corfas and Dudai, 1989). A good example of nonassociative behaviour is habituation and

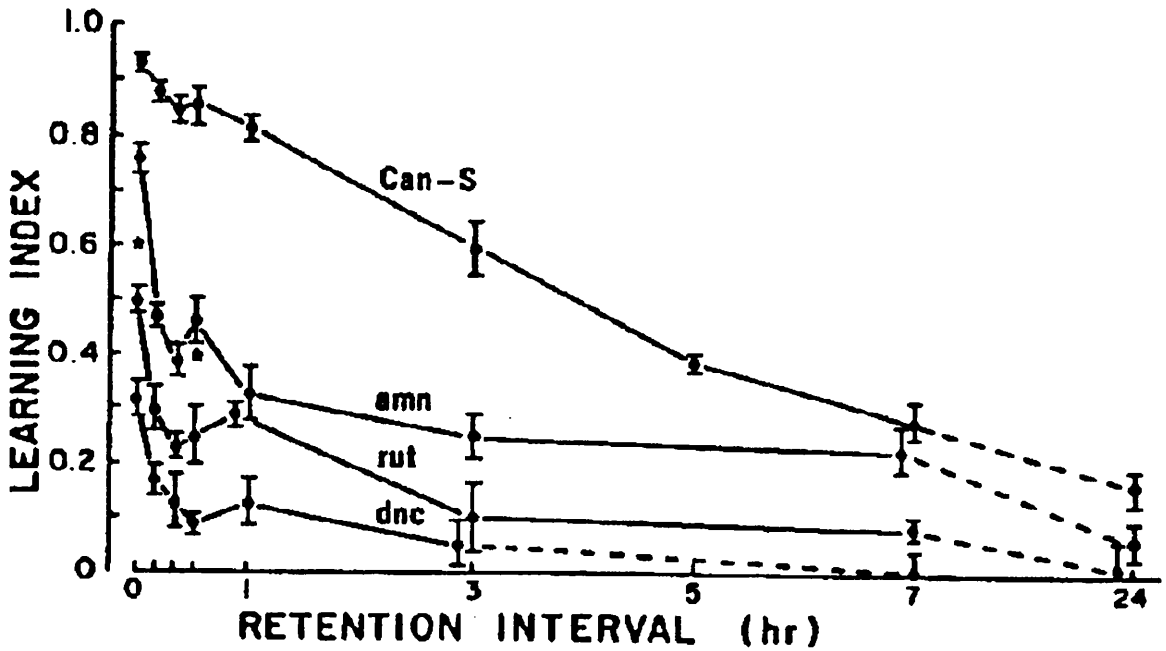


Figure 1.2. Memory retention in normal and mutant flies. Different groups of flies received 12 shock pulses during training and then were tested at various intervals afterwards. Retention curves are drawn for wild-type (Can-S), *amnesiac* (amn), *rutabaga* (rut), *dunce* (dnc) flies. Each point represents 4 experiments. (Taken from Tully, 1991).

dishabituation of the cleaning reflex (Corfas and Dudai, 1989). Mechanical stimulation of single, or a set of, thoracic bristles elicits characteristic movements of the appropriate leg directed to clean the thoracic area of the bristles. The cleaning response is habituated and ultimately disappears upon repeated stimulation. Dishabituation can be achieved by high frequency stimulation of another set of bristles on the thorax.

This is by no means a exhaustive list of the behaviours and paradigms for demonstrating learning in *Drosophila melanogaster.* However, it hopefully highlights the extensive repertoire of behaviours which can be studied. More importantly, they have allowed the isolation of mutations affecting these processes.

1.3 Mutants

It is important to define what a learning mutant is and to know whether the majority of genes or only a small subset, in one way or another, affect learning and memory Many mutations may be expected to interfere with behavioural plasticity. Moreover, most of these mutations might also be expected to interfere with development, metabolism, and sensory or motor capabilities. So do pure learning mutants exist at all? Dudai, 1988 highlights this problem and sets operational criteria for a learning mutant: "The mutation should affect acquisition and/or retention and/or retrieval, but the effect on these processes should be reasonably dissociated, under the appropriate experimental conditions, from the effects on other functions of the organism". The range of experimental paradigms which exist to assess flies ability to learn and remember have allowed the isolation of mutations affecting these processes. Each paradigm has its own inherent advantages and disadvantages. The first set of learning mutants was isolated after chemical mutagenesis with ethyl methanesulfonate (EMS) and selected by quantifying the performance of EMS-treated flies in the olfactory negative reinforcement test described earlier (Quinn et al., 1974 ; Dudai et al., 1976; Quinn et al., 1979; Aceves-Pina et al., 1983). The mutagenesis and breeding scheme used in these experiments selected specifically for mutations on the X-chromosome. Using this approach, six

mutant strains were isolated; *dunce*, *rutabaga*, *radish*, *turnip*, *cabbage*, and *amnesiac* (Dudai et al., 1976; Quinn et al., 1979; Aceves-Pina et al., 1983). Although this strategy is highly mutagenic, single base changes generated by EMS mutagenesis are difficult to map genetically, leaving the gene of interest difficult to define at the molecular level. In fact, genetic mapping experiments have only established that the behavioural effects of the *dunce*, *rutabaga*, and *amnesiac* mutations result from single genes (Aceves-Pina and Quinn, 1979; Quinn et al., 1979; Booker and Quinn, 1981; Byers et al., 1981; Livingstone, 1985; Tully and Gergen, 1986). In addition, several EMS-induced mutations on the X or other chromosomes, which were isolated because of structural defects in the brain, were found to be defective in specific learning paradigms (Heisenberg et al., 1985). This provided evidence that certain structures, notably mushroom bodies (MBs), were involved in *Drosophila* olfactory learning. In an effort to extend the isolation of learning and memory genes and alleviate the problems associated with analysing EMS-induced mutations, a number of new approaches have been taken. These include 'enhancer-trapping' (see section 1.8.6) techniques to isolate new mutants based on behavioural or 'anatomically preselected' phenotypes (Boynton and Tully, 1992; Han et al., 1992).

Returning to important issues raised earlier in this introduction; does a mutation affect associative learning (or memory) *per se*? For example, mutants isolated in a olfactory negative reinforcement screen could easily have disruptions in 'peripheral' behaviours such as olfaction and/or mechanoreception. An example of such a mutant is *smellblind*. This mutant was originally isolated in a screen for mutants defective in an olfactory associative learning paradigm (Aceves-Pina and Quinn, 1979). It was found subsequently to be defective in olfactory response at both the larval and adult stages. Fortunately, this sort of problem can be addressed to some extent: firstly, naive flies can be carefully assessed for these 'peripheral' behaviours and secondly, the mutants can be tested in more than one paradigm, using different sensory modalities. It is clear that there are two classes of learning mutants, those that interfere with the basic development of the nervous system and those that affect the efficacy of synaptic transmission. A

potentially intractable problem is, do the mutations that interfere with physiological processes mediating learning also affect the basic development of the nervous system?

In the following section I will describe some of the genes from both classes, that affect *Drosophila* learning and memory processes (see Table 1).

1.3.1 Biochemical learning mutants

dunce

The first mutant isolated in a search for learning mutants using an olfactory negative reinforcement paradigm was named *dunce* (*dnc*) (Dudai et al., 1976). Two *dnc* alleles, dnc^1 and dnc^2 were isolated in this screen. When trained and tested in the original olfactory negative reinforcement paradigm, *dnc* learned poorly. When examined more carefully in a slightly modified olfactory avoidance paradigm, *dnc* also showed initial learning slightly lower than that of wild-type and a very labile memory (Dudai, 1979 ; Dudai, 1983). Different results were obtained in an olfactory reward learning paradigm (as described above and in Tempel et al., 1983). *dnc* flies showed normal acquisition but the memory decayed much faster than that of wild-type. In a classical conditioning paradigm, Tully and Quinn (1985) showed that *dnc* flies were capable of initial learning at an intermediate level (relative to wild-type) when tested intermittently after training. In comparison to the progressively decaying memory exhibited by wild-type flies, the memory retention of *dnc* flies decayed very rapidly during the first half hour of training. These experiment cannot address whether the *dnc* mutation affects acquisition specifically, or whether it affects the short-term memory which decreases too rapidly to be tested. Therefore, it may be concluded that *dnc* is either an acquisition mutant, or a short-term memory mutant, or both.

In addition to the paradigms described above, the *dnc* flies behaved poorly in another associative learning paradigm, experience-dependent modification of courtship. Courtship in *Drosophila* involves a complex series of behavioural acts, in which a variety

Gene	Gene Product	Negatively Reinforced Olfactory Learning	Positively Reinforced Olfactory Learning	Larval Olfactory Learning	Expression Pattern	Method of isolation	Other phenotypes	References
dunce (*dnc*)	cAMP PDE	Poor learning	Normal learning; poor memory	Poor learning	Preferential MB expression	EMS-behaviour; female sterile	female sterility	Dudai et al., 1976 Aceves-Pina and Quinn, 1979; Dudai, 1983
rutabaga (*rut*)	Adenylate cyclase	Poor learning	Poor learning		Preferential MB expression	EMS-behaviour; enhancer trap; EMS-suppressor of *dnc* sterility	A weak suppressor of *dnc* female sterility	Dudai, 1983; Tempel et al., 1983; Livingstone et al., 1984; Tully and Quinn, 1985; Bellen et al., 1987; Levin et al., 1992; Han et al., 1992
DCO (*DCO*)	catalytic subunit of PKA	Poor learning			Preferential MB expression	EMS-lethal; enhancer trap	lethal	Skoulakis et al., 1993 Lane and Kalderon, 1993
mushroom body deranged (*mbd*)		Poor learning	Poor learning	Normal learning		EMS-anatomy	Anatomical defect of MBs	Heisenberg et al., 1985
mushroom body miniature (*mbm*)		Poor learning	Poor learning	Poor learning by females; normal learning by males		EMS-anatomy	Sexually dimorphic anatomical defect of MBs	Heisenberg et al., 1985
amnesiac (*amn*)		Poor learning	Normal learning; poor memory	Normal memory		EMS-behaviour		Aceves-Pina and Quinn, 1979; Quinn et al., 1979; Tempel et al., 1983
cabbage (*cab*)		Poor learning		Poor learning		EMS-behaviour		Aceves-Pina and Quinn, 1979;Booker and Quinn, 1981; Dudai, 1983
turnip (*tur*)	Involved in PKC activity	Poor learning	Poor learning	Poor learning		EMS-behaviour		Aceves-Pina and Quinn, 1979; Quinn et al., 1979; Tempel et al., 1983; Choi et al., 1991
radish (*rad*)		Poor learning				EMS-behaviour		Folkers et al., 1993
latheo (*lat*)		Poor learning				Transposon-behaviour	Lethality Structural mutant?	Boynton and Tully, 1992 Boynton (pers comm)
linotte (*lio*)		Poor learning				Transposon-behaviour	Possibly central complex defects	Dura et al., 1993 Préat (pers comm)
Protein Phosphatase 1	Protein Phosphatase 1	Poor learning					Reduced viability	Asztalos et al., 1993

Table 1. Summary of genes involved in olfactory learning. (Adapted from Davis, 1993).

of sensory modalities are exchanged between the partners in preparation for copulation. Most of the stages in courtship are innately programmed, but some responses are modifiable by experience. The experience may act as either a positive or negative reinforcement. An example of a negative reinforcement is the depression of the males courtship following rejection by a fertilised female (Hall, 1986). The rejected male avoids any female, including receptive virgins, for some hours afterwards. However, *dnc* males court females at a frequency significantly higher than wild-type even after prior experience of a fertilised female (Gailey et al., 1984). Intriguingly, this apparent need for experience-dependent modification in courtship has led to the hypothesis that learning may have evolved to contribute to efficient reproduction (Hall, 1986).

It is notably that *dnc* mutants are also defective in another nonassociative learning paradigm, habituation and sensitisation of a proboscis-extension feeding reflex (Duerr and Quinn, 1982). Thus mutations in the *dunce* gene affect processes that are common to both associative and nonassociative learning.

dnc mutants have been isolated independently as cAMP phosphodiesterase (PDE) mutants. Two major forms of cyclic nucleotide phosphodiesterase (I,II) and one minor form (III) of PDEs have been found in the wild-type adult fly (reviewed by Kiger and Salz, 1985). Form I is a heat-stable Ca^{2+}/ Calmodulin-sensitive PDE, hydrolysing both cGMP and cAMP. Form II is a moderately heat-labile cAMP specific PDE (Davis and Kiger, 1980; Kauvar, 1982). Form III is an extremely heat-labile cGMP specific PDE (Kauvar, 1982). In a series of elegant experiments, assaying cyclic nucleotide hydrolysis in segmental aneuploids and in flies carrying deficiencies and/or duplications, Kiger and co-workers assigned the form II activity to cytogenetic region 3D4 on the X chromosome (Kiger and Golanty, 1977; Kiger and Golanty, 1979; Kiger and Salz, 1985).

In addition, two female sterile mutants (dnc^{M11} and dnc^{M14}) were isolated from a screen for female sterile mutations (Mohler, 1977), complementation experiments demonstrated that both mutants were deficient in form II PDE activity and therefore alleles of *dnc* (Byers et al., 1981). Later, it was shown that dnc^{M11} and dnc^{M14} had learning defects (Tully, 1991). Two additional mutants were isolated by Kiger et

al.(1981) *dnc*^{ML} was recovered as an amorph, having no apparent cAMP PDE activity and *dnc*^{CK} isolated because of its female sterility. All six *dnc* mutations (*dnc*^{1}, *dnc*^{2}, *dnc*^{M11}, *dnc*^{M14}, *dnc*^{ML} and *dnc*^{CK}) are allelic (Kiger et al., 1981) and apart from *dnc*^{CK}, which is a chromosomal translocation, the other five alleles are point mutations.

Biochemical evidence and gene-dosage studies suggested that *dnc* is the structural gene for cAMP PDE. Firstly, all six *dnc* alleles (in hemizygous males or homozygous females) have diminished or no cAMP PDE activity (Davis and Kiger, 1981). *dnc*^{1}, *dnc*^{2} and *dnc*^{CK} have 40% to 50% of the cAMP PDE activity relative to wild-type and *dnc*^{M11}, *dnc*^{M14}, and *dnc*^{ML} are amorphic alleles, producing no cAMP PDE activity. From gene-dosage experiments, it is clear that flies lacking cytogenetic region 3D4, not only lack cAMP PDE and are sterile (Kiger and Golanty, 1979), but that no change in activity can be detected for the other forms of PDE (Davis and Kiger, 1981; Shotwell, 1983). Unequivocal evidence came from the cloning of the *dunce* gene (Chen et al., 1986; Qiu et al., 1991). The gene is very complex, it extends over 148 kb, contains at least 19 exons, and encodes at least 10 RNAs (ranging in size from 4.2 to 9.6 kb) with perhaps as many as seven different PDE isoforms by alternative splicing and multiple transcription start sites (Qui et al., 1991; Qui and Davis, 1993). It would appear that the use of different transcription start sites confers multiple biological functions. One is principally responsible for directing elevated mushroom body expression, while others are required for the female fertility and learning functions (Qui and Davis, 1993). Immunohistochemical studies have shown that the *dnc* PDE is concentrated in the mushroom body (MB) neuropil (Nighorn et al., 1991). The expression is not specific, as the *dnc* PDE is also found at much lower levels in other neuropil areas. RNA *in situ* hybridisation has shown that *dnc* RNAs are enriched in mushroom body perikarya over other neurons (Nighorn et al., 1991). This suggests that the high level of expression in the mushroom bodies is due at least in part to transcriptional or post-transcriptional control. These findings are consistent with other lines of evidence that suggest that MBs are crucial for information processing and integration in insects (see Chapter 4 ; Erber at al., 1980; Heisenberg, 1985; de Belle and Heisenberg, 1993). However it must be

stressed that it is still unclear if elevated MB expression of the *dnc* gene is important for learning and memory processes.

rutabaga

A second mutant, *rutabaga* (*rut*), also involved in cAMP metabolism has been implicated in *Drosophila* learning and memory. Like dnc^1 and dnc^2 a mutant allele of *rut* was isolated by the olfactory associative learning paradigm using the same selection criteria (Aceves-Pina et al., 1983; Dudai et al., 1984; Dudai, 1988). Like *dnc* mutants *rut* flies learn less well than wild-type in olfactory conditioning and are incapable of remembering it normally. The *rut* mutation decreases the level of adenylate cyclase (AC) in *Drosophila* by about 25% (Dudai et al., 1983; 1984; Livingstone et al., 1984). The mutation abolishes the stimulation of adenylate cyclase by Ca^{2+}/calmodulin (Dudai and Zvi, 1984; Livingstone et al., 1984; Livingstone, 1985). The mutation causing the enzymatic defect as well as the learning defect maps to the X-chromosome, cytogenetic region 12F5-7 (Dudai et al., 1985; Livingstone et al., 1984; Livingstone, 1985). Recent cloning and expression of the *rutabaga* gene proved that the gene encoded a Ca^{2+}/calmodulin-sensitive adenylate cyclase (Levin et al., 1992).

In addition to the convergence in behavioural and biochemical defects of the *dnc* and *rut* mutants, Han et al. (1992) showed that there was a neuroanatomical convergence of expression patterns. Immunohistochemical and RNA *in situ* hybridisation studies, like *dnc*, show that expression of *rut* is significantly elevated in the MBs of wild-type flies. From an enhancer-trap screen for 'anatomically preselected' phenotypes, i.e. genes preferentially expressed in the MBs, 7 unique alleles of *rut* were recovered (Levin et al., 1992; Han et al., 1992). Interestingly, the severity of the learning phenotypes associated with each insertion line seems to be correlated with the expression level of AC in the MBs as well as with the proximity of the insertion relative to the putative transcription start site.

cAMP-dependent Protein kinases

The major effector enzyme of cAMP, the cAMP-dependent protein kinase (PKA), is composed of two regulatory (R) and two catalytic (C) subunits forming the inactive tetrameric enzyme R_2C_2. Binding of cAMP to the regulatory subunits causes dissociation of the holoenzyme into free regulatory and free (active) catalytic subunits. The catalytic subunit is then free to phosphorylate substrate proteins on a serine or threonine residue preceded by two basic residues, usually arginines (Taylor et al., 1990). Experiments by Drain et al. (1991), provided compelling evidence that PKA has a direct role in olfactory learning and memory in *Drosophila*. They showed that olfactory learning was disrupted in transgenic flies containing a peptide inhibitor of PKA. More recently, two recessive lethal mutations in *DCO*, the catalytic gene for PKA have been isolated. One is a recessive lethal point mutation, DCO^{B10}, the second, DCO^{581} was isolated by an 'enhancer-trap' screen for genes preferentially expressed in the MBs (Lane and Kalderon, 1993; Skoulakis et al., 1993). Both the DCO^{B10} and DCO^{581} alleles are homozygous lethal and heterozygotes have a 40% reduction in PKA activity. This reduction has subtle effects on learning but no effect on memory. However, a heteroallelic combination results in viable flies with an 80% reduction in PKA activity. These flies had obvious defects in learning, but peculiarly, stable memory remained in these heteroallelics with very low levels of PKA activity (Skoulakis et al., 1993). Like *dnc* and *rut*, the *DCO* gene plays a crucial role in the processes of olfactory learning and memory and is also preferentially expressed in the MBs, again suggesting that these cells may mediate behavioural plasticity.

More recently it has been shown that flies deficient for Protein Phosphatase 1 were defective in both nonassociative and associative behaviours (Asztalos et al., 1993). This is consistent with the above observations, that mutations affecting cAMP-dependent phosphorylation disrupt the learning processes of *Drosophila*.

Other mutants obtained in behavioural screens

To date, only olfactory associative learning has been used as a behavioural screen for learning mutants. Six additional olfactory learning mutants have been recovered: *turnip* (*tur*), *amnesiac* (*amn*), *cabbage* (*cab*), *radish* (*rsh*), *latheo* (*lat*) and *linotte* (*lio*) (Quinn et al., 1979; Boynton and Tully, 1992; Dura et al., 1993).

In the shock-odour associative learning paradigm, the *turnip* mutation is recessive for initial learning, but dominant for memory retention; the initial learning of *tur*/+ is near normal, yet its memory decays very rapidly (Quinn et al., 1979). The mutation reduces the activity of protein kinase C (PKC) by 70-80% and in addition, *tur* flies are deficient in phosphorylation of a 76 kDa head membrane protein, which is a major substrate for PKC in wild-type homogenates (Choi et al., 1991). Surprisingly no known PKC genes map to the *tur* locus and it is not clear whether all the biochemical and behavioural defects associated with the mutant actually map to a single locus.

The remaining mutants isolated all have defects in learning and memory in one or more learning paradigms (Aceves-Pina and Quinn, 1979; Quinn et al., 1979; Duerr and Quinn, 1982; Tempel et al., 1983; Tully, 1991; Boynton and Tully, 1992; Dura et al., 1993). The molecular basis of these mutations is unknown, but in the case of *latheo* and *linotte*, the gene of interest may be "tagged" with a P-element, alleviating subsequent molecular cloning (Boynton and Tully, 1992; Dura et al., 1993).

In *Drosophila*, memory is multiphasic, i.e. an early rapid decay phase of several minutes followed by a slow decay phase, which may last for many hours (Tully and Quinn, 1985; Tully, 1991). Early work by Quinn and Dudai (1976), indicated that the formation of multiple memory phases is best illustrated by the use of cold anaesthesia. When wild-type flies are cooled in ice water during the first few minutes after training in a classical olfactory conditioning paradigm, their subsequent memory is reduced significantly. In comparison, cooling flies 30-120 min after training did not affect the memory significantly. The anaesthesia-sensitive memory phase was indicative of 'short-term' memory, and the anaesthesia-resistant memory phase was indicative of 'long-term'

memory. The mutants *amn* and *rsh* behave quite differently in the above test. Both mutants have good initial learning, with rapid decay of memory within the first hour of training (Tully et al., 1990; Folkers et al., 1993). In the *amn* mutant memory is steady and persists for at least 8 hours, in contrast, memory in *rsh* mutants is completely gone by 6 hours. Furthermore, *amn* can form anaesthesia-resistant memory, whereas *rsh* cannot. Thus, *rsh* may be defective in consolidation of memory.

1.3.2 Structural mutants

Many mutants showing abnormal brain structures have been isolated by Martin Heisenberg's laboratory (Heisenberg and Boehl, 1979; Fischbach and Heisenberg, 1984; Heisenberg, 1989). These mutants can be classified into four different groups based on the affected brain structures; mushroom body mutants, general brain hypoplasia mutants, optic lobe mutants, and central complex mutants. In *Drosophila*, as in vertebrates, evolution has resulted in cephalization of the nervous system into the head. Four distinct areas are present in the fly head; visual, olfactory, gustatory and mechanosensory (Power, 1943; Strausfeld, 1976). The fly brain is often divided into two divisions, the upper and lower brain. The upper brain consists of four parts; protocerebrum, deuterocerebrum, tritocerebrum and optic lobes. The protocerebrum is made up of many structures such as the central body, mushroom bodies and protocerebral bridge. The deuterocerebrum can be divided into antennal lobes, the antennal mechanosensory regions and other areas for sensory inputs and descending outputs. The lower brain covers mouthparts, neck and salivary glands.

Mushroom bodies (MBs)

Mushroom bodies are bilateral clusters of about 2500 cells, situated in the dorsal and posterior cortex of each brain lobe (Figure 1.3). The cells send dendrites into neuropil (space in the CNS occupied primarily by dendrites and axons) just ventral to the

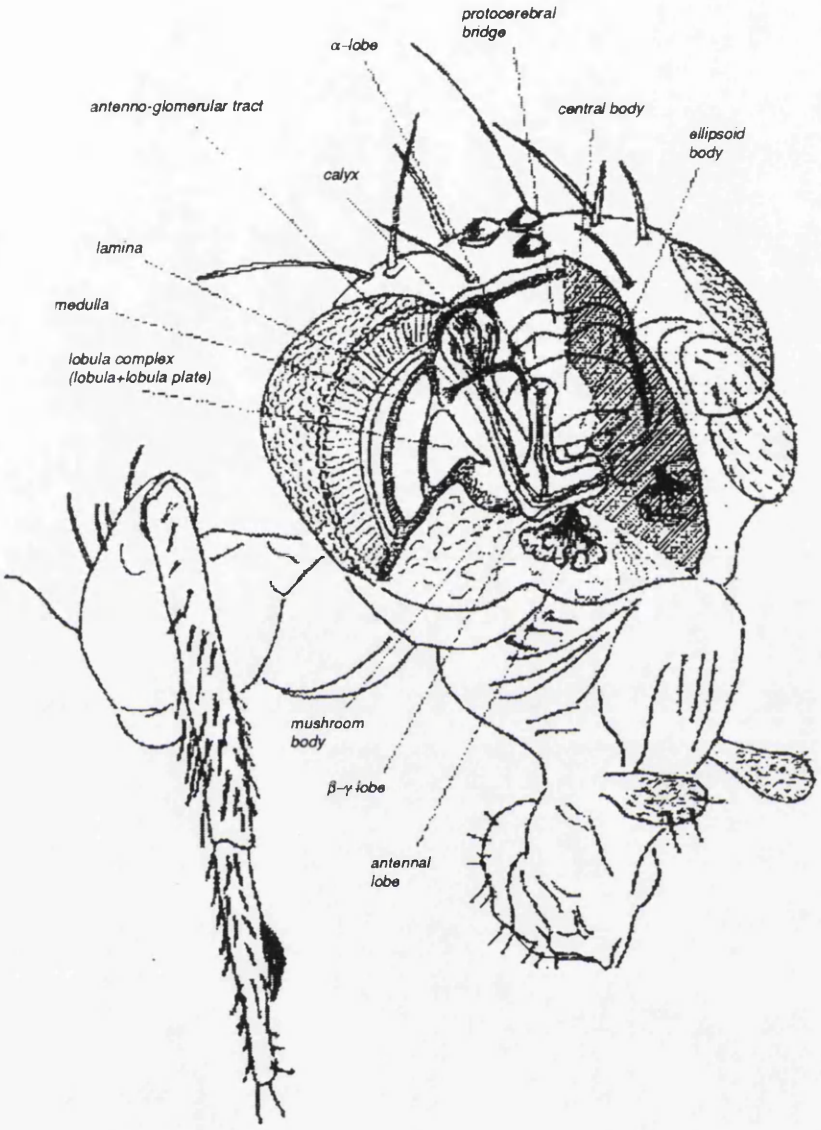


Figure 1.3. Schematic drawing of an opened head of *Drosophila* showing the most prominent parts (enlarged) of its central brain. (Taken from Heisenberg et al., 1985).

perikarya (cell body of neuron, excluding processes) into the calyx (a region for dendritic arborisation). The axons of the MBs (pedunculi) extend anteriorly and slightly ventrally. At the ventral margin of the protocerebrum, near to its anterior surface, each pedunculus splits sending some processes medially to form the neuropil region known as the β and γ-lobes, and some extend dorsally to form the α-lobe.

The MBs have been implicated in olfactory learning in insects for a number of reasons. Firstly, Erber et al., 1980, showed that selective cooling of the MBs in the bee causes memory loss. Secondly, Heisenberg and Boehl, (1979) have isolated two mutants with defective mushroom body anatomy, *mushroom body deranged* (*mbd*) and *mushroom body miniature* (*mbm*), each of which has poor olfactory learning (Heisenberg et al., 1985). The *mbm* mutation is of particular interest, causing a sexually dimorphic disruption of the MBs. Mutant males have normal MBs and learn normally as larvae and adults. In contrast, females have no discernible MBs and display poor learning as larvae and adults. Thirdly and more recently, in an effort to alleviate the problems associated with the unknown ætiology and specificity of MB defects, de Belle and Heisenberg, (1993) have used a non-genetic approach to generate mushroom body-ablated flies. By feeding hydroxyurea to newly hatched larvae they selectively killed the four MB neuroblasts that are the precursors for the MBs. Adult flies devoid of MBs behave normally, but are unable to perform in a classical olfactory conditioning paradigm. Lastly, since sensory information from several sources is known to converge on the MBs, the MBs are thus plausible sites to receive and process information from different sensory modalities (Heisenberg, 1989).

Other structural mutants

In the centre of the brain neuropil, between the two MB pedunculi and above the oesophagus lies the central complex, a regular array of repetitive fibres. It consists of four substructures, protocerebral bridge, fan-shaped body, ellipsoid body and the noduli.

Several structural mutants with defects in the central complex, *central body defect* (*cbd*), *no bridge* (*nob*), *ellipsoid body open* (*ebo*), *central complex broad* (*ccb*) and *central complex deranged* (*ccd*), with the exception of *ebo*, are impaired in olfactory conditioning (Heisenberg et al., 1985). However, general behaviours of these mutants has not been studied in detail, but the mutants appear to have reduced locomotor activity (Heisenberg, 1989) and are impaired in walking activity and walking speed (Strauß and Heisenberg, 1993). Thus, it is unclear whether the behavioural impairment of these mutants in olfactory learning reflects a role of the central complex in olfactory learning or a defect in general locomotion.

1.4 Why do the mutants have learning and memory defects ?

In *Drosophila*, genetic dissection of learning and memory has shown that mutations affecting the cAMP signal transduction pathway, and probably other second-messenger pathways, disrupt olfactory learning and short-term memory. For example, one explanation of the observed behaviour of *dnc* and *rut* flies is that abnormal levels of cAMP, too high or too low, or the inability to control cAMP levels during training, produce reductions in learning and short-term memory. Since the *dnc* and *rut* mutations influence cAMP metabolism in opposite directions, the possibility arises that normal learning requires a certain optimal range of cAMP levels. One must assume that cAMP is acting physiologically during training or shortly thereafter. An alternative explanation should, however, be considered. It is possible that the abnormal behaviour is caused by developmental defects, resulting in neuroanatomical aberrations, e.g. neuronal wiring. At least for *dunce*, this problem has been addressed by introducing a construct carrying a *dunce* cDNA clone, driven by a heat shock promoter, into *dunce* flies. Upon heat shock, the transgenic flies learn near normally (Dauwalder, E.S.D.N, Glasgow, 1992). Thus for *dunce* the major requirement is a physiological one, rather than a purely developmental requirement. In addition, flies fed with pharmacological inhibitors of cAMP metabolism, mimic learning and memory problems associated with these mutations

(Folkers and Spatz, 1984; Asztalos et al., 1991). It would seem unlikely that such treatments would cause morphological changes in the adult nervous system. There is no doubt that the cAMP signal transduction pathway plays an important role in development (Lane and Kalderon, 1993). However, the important point is that the developmental effects can be dissociated from the effects on learning. In addition to the *dnc* and *rut* mutations, defects in other components of cAMP metabolism, namely *DCO* and protein phosphatase 1 affect learning and memory processes and point to cAMP-dependent protein phosphorylation in the neuromodulatory mechanisms underlying *Drosophila* learning and memory.

This is in accordance with the physiological findings from studies into learning and memory in the sea snail, *Aplysia californica* (Kandel, 1991). Kandel and Schwartz, (1982), proposed a model of facilitation and sensitisation of the gill-siphon withdrawal reflex, that was mediated by the cAMP signal transduction pathway. Sensitisation at the electrophysiological level is associated with facilitation of synaptic transmission between sensory and motor neurons mediating the reflex. One possible mechanism for facilitating transmission is initiated via the release of a mono-amine neurotransmitter at a "facilitatory" synapse near to the pre-synaptic terminal of a sensory neuron causing activation of a membrane receptor of the pre-synaptic neuron. This in turn activates, via a G-protein, adenylate cyclase which increases presynaptic levels of cAMP. At increased levels of cAMP, the cAMP-dependent protein kinase (PKA), inactivates a K^+ channel, thus broadening the action potential, resulting in prolonged influx of Ca^{2+} and increased neurotransmitter release. Repeated stimulation of the reflex causes a long lasting facilitation of synaptic transmission that requires protein synthesis and may be maintained by the persistent activation of PKA caused by a prolonged decrease in the concentration of regulatory subunits of the kinase. The model was extended by Hawkins et al., (1983) to explain classical conditioning, by conferring on the AC the ability to be activated by Ca^{2+}/Calmodulin (Figure 1.4). They showed that simultaneous activation of AC by the facilitatory transmitter and by free intracellular Ca^{2+} was larger than the sum of the two activation effects applied non-coincidently. Hence, the models of

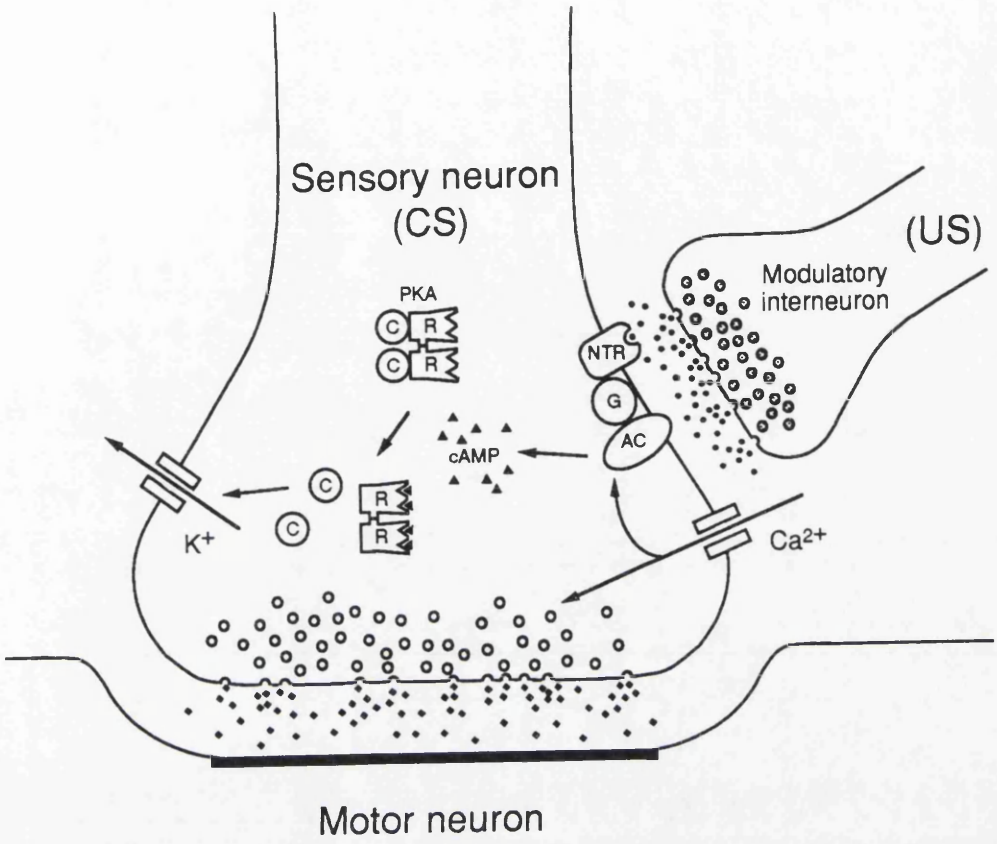


Figure 1.4. Model of associative learning in the *Aplysia* gill-siphon withdrawal reflex. The unconditoned stimulus (US; shock) activates adenylate cyclase via a modulatory interneuron, receptor (NTR) and G-protein (G). Prior stimulation of the sensory neuron by the conditioned stimulus (CS; tactile stimulus) coactivates adenylate cyclase by Ca^{2+} causing an increase in [cAMP]. This in turn activates a cAMP-dependent protein kinase which phosphorylates and inactivates a K^+ channel in the sensory neuron, thus broadening the action potential, resulting in prolonged influx of Ca^{2+}, leading to more transmitter release in response to stimulation (CS) of the sensory neuron alone. (Diagram taken from Sentry et al., 1993).

nonassociative and associative learning share aspects of the same secondary messenger cascade. This also parallels the findings for behavioural studies of *dnc* and *rut*. However, two observations contradict this. Firstly, *rut* is defective in nonassociative behaviours and the *Aplysia* model does not predict a role for Ca^{2+}/Calmodulin activation of adenylate cyclase in habituation and sensitisation. Secondly, as Tully (1991) points out, if the Ca^{2+}/Calmodulin adenylate cyclase is indeed the point of convergence of the conditioned stimulus (CS) and the unconditioned stimulus (US), why do *rut* flies exhibit any associative behaviour at all? Thus, for associative learning in *Drosophila*, the picture may be far from complete, and maybe indicates that other second messenger systems are involved as well. In fact several researchers have proposed alternative models for associative learning in *Drosophila* (Aszódi and Friedrich, 1987; Buxbaum and Dudai, 1989; Müller and Spatz, 1989; Friedrich, 1987; 1990; Aszódi et al., 1991). All of the models suggest that signal convergence on type II protein kinase A (PKA) may act as a molecular correlate of learning. Two types of mechanism could be considered for this purpose; (a) A second messenger-induced modification of the kinase increases its sensitivity to the second messenger, so that low concentrations of the latter, previously ineffective, now suffice to cause dramatic activation of the enzyme. (b) The active kinase becomes 'autonomous' of its second-messenger. In PKA, in the absence of cAMP, the regulatory subunit is coupled to the catalytic subunit, inhibiting its kinase activity. Upon binding of cAMP, the regulatory subunit dissociates from the catalytic subunit, hence activating it (reviewed by Nestler and Greengard, 1984). Various processes can lead to enduring changes in the interaction between the two subunits, as mentioned earlier, autophosphorylation of the regulatory subunit endows the enzyme with the property of prolonging the cAMP effect (Buxbaum and Dudai, 1989), or selective proteolysis of the R subunit can elevate kinase activity (Greenberg et al., 1987). Another model invokes the Ca^{2+}-activated neutral proteinase calpain in the proteolytic modification of the RII subunit of *Drosophila* PKA (Müller and Spatz, 1989). All the above studies point to a model of neuronal plasticity in which cellular signals converge on PKA.

1.5 Conclusions from the genetic dissection of learning and memory

Drosophila, with its rich history of classical genetics, molecular techniques and its ability to exhibit both associative and nonassociative behaviours, have made it an ideal organism for the genetic dissection of learning and memory. Genetic screens adopting various strategies have isolated a series of olfactory learning mutants. The mutants *dnc*, *rut* and *DCO* are involved in the cAMP signal transduction pathway, influence olfactory learning and show preferential expression in the MBs. The main heuristic conclusion that can be drawn proposes that MBs are the principal sites mediating olfactory learning via the cAMP second messenger pathway. At the network level, *mbm* and *mbd* mutants suggest a role of the mushroom bodies in olfactory learning. These two genes may serve important roles in the structural plasticity of the MBs (Technau, 1984).

Convergence of results from *Drosophila* and *Aplysia* emphasises the similarity of the cellular biochemistry thought to underlie associative and nonassociative learning. However, the target(s) for both signal convergence and signal extension remains to be elucidated. Furthermore, the fact that no totally "stupid" flies have been isolated by conventional behavioural screens, may reflect that there is no exclusive mechanism of learning and short-term memory, but rather several second messenger systems and neural centres operate in parallel.

1.6 Limitations of the genetic dissection of learning and memory

To date, only a handful of genes have been isolated that affect olfactory learning and memory. Failure during the last decade to provide further insights into the molecular identity for most of these genes raises a fundamental question, whether the approaches taken so far have been successful? The traditional approach, based on behavioural screening of chemically mutagenised flies was limited to the X chromosome, offered a high rate of mutagenesis and allowed the isolation of any gene required for learning that was nonessential. The X chromosome comprises only about 20% of the *Drosophila*

genome. The rest of the genome has remained virtually untouched in terms of searching for learning and memory genes. In addition, mapping and cloning genes identified by point mutations is not trivial. It is therefore no surprise that researchers have used a variety of new approaches, including 'enhancer-trapping', to isolate new mutants based on behavioural or 'anatomically preselected' phenotypes.

The detailed knowledge of the molecular components of the cAMP signal transduction pathway make it an attractive system for manipulation by reverse genetical approaches. In particular, all of the *Drosophila* G proteins have been cloned without reference to phenotype, by homology probing (Buchner, 1991).

1.7 P-element Biology

P-elements are a family of transposable elements found in *Drosophila melanogaster.* They have been shown to be the causal agents of P-M hybrid dysgenesis, a syndrome whose traits include high rates of sterility, mutation, and chromosomal rearrangements (Engels, 1988; Engels, 1989; Rio, 1990). P-element transposition is genetically regulated, occurring at very high frequency only in the progeny from a cross in which P-element containing males (P strains) are mated to females lacking P-elements (M strains). No dysgenic traits are observed in the progeny of the reciprocal M male by P female cross or in the progeny from P x P or M x M crosses. Moreover, transposition is restricted to cells of the germ-line. Thus, P-element transposition is regulated in two ways: genetically and tissue specifically. The distinguishing characteristic of P strains is that they contain full-length autonomous P-elements, encoding their own transposase. P strains also contain defective, generally internally deleted, P-elements. Transposition in a P strain is tightly regulated. It is repressed by a product of the full-length P-element itself. This condition is known as "P cytotype" (Engels, 1979). M strains, in comparison, lack autonomous P-elements and are permissive for P-element transposition ("M cytotype"). Thus, transposition and hybrid dysgenesis are induced when potentially active P-

elements are introduced into the permissive cellular environment of M cytotype which is transmitted by the female parent.

Molecular analysis indicated that the P-elements present in P strains could be divided into two classes; a class of full-length 2.9 kb elements that encode two different proteins depending on the pattern of pre-mRNA splicing (Figure 1.5) and a heterogeneous class of internally deleted elements, some of which appear to encode truncated proteins e.g. KP element (Figure 1.5; O'Hare and Rubin, 1983; Black et al., 1987). All P-elements have 31 bp terminal inverted repeats and sequences required in *cis* for transposition are contained within 138 bp at the 5' end and 150 bp at the 3' end. Internally deleted elements retain *cis* -acting determinants that allow mobilisation in the presence of a functional transposase source. Full-length elements have four open reading frames encoding an 87 kDa transposase (Figure 1.5; Karess and Rubin, 1984). Restriction of P-element activity to the germ-line results from differential splicing of the mRNA such that the third (2-3) splice occurs only in the germ-line (Laski et al., 1986) An engineered P-element with the third intron removed (Δ2-3) is unable to transpose in somatic cells, and in addition lacks the ability to establish P cytotype (Figure 1.5; Laski et al., 1986).

When P-elements transpose they leave behind a double-stranded break that is frequently the subject of widening by exonucleases, and the repair of which appears to require a template (Figure 1.6; Engels et al., 1990; reviewed by Sentry and Kaiser, 1992). Excision of the P-element can either be 'precise', when the donor site is restored to its wild-type sequence, or result in specific parts of the element remaining at the former site, i.e. 'imprecise' excision. Studies performed by Engels et al., 1990, suggest that a double-stranded break repair model could explain precise and imprecise excisions (see section 1.8.4). According to this model, sister chromatids or homologous chromosomes of the broken molecule are used as templates for repair. If the template contains the P-element, double stranded repair will mostly produce a chromosome identical in appearance to the donor chromosome prior to transposition. In a few cases, however, repair can be interrupted, resulting in the generation of nonautonomous P-element deletion derivatives. A

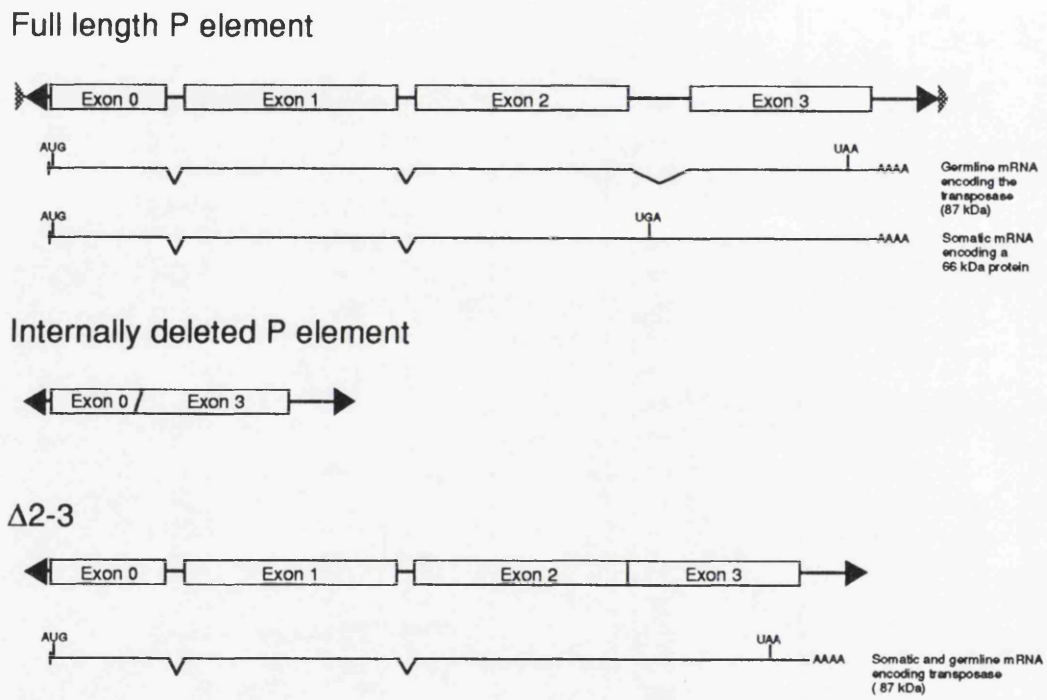


Figure 1.5. Structure of the full length 2.9 kb P-element, internally deleted P-element and Δ2-3 element. The terminal 31 bp inverted repeat and the direct 8 bp target site duplication are denoted by arrow heads (black and shaded, respectively). Translation initiation and termination codons are shown for the full length P-element and the Δ2-3 element. (Diagram taken from Sentry and Kaiser, 1993)

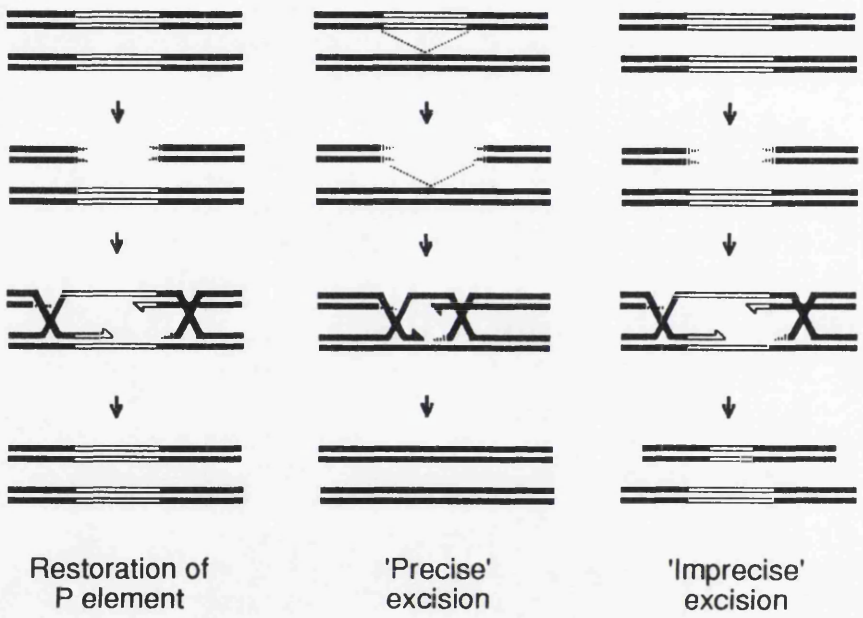


Figure 1.6. Model for template-dependent gap repair following P-element excision. Excision of a P-element (open bars) induces a double-strand break that can be subject to widening by exonucleases. Free 3' ends invade the template duplex, which serves as a substrate for DNA synthesis. In the left panel, the template is a second copy of the P-induced allele, most commonly provided by a sister chromatid. The result is restoration of a P-element at the locus. Less frequently, the template can be a wild-type allele present on an homologous chromosome (centre panel). This will give the impression of precise excision. Interruption of the repair process, in this case where the sister chromatid is the template, followed by pairing of partially extended 3' ends, may give the impression of an 'imprecise excision' (right panel). This can take the form of internal deletion of the P-element, or more extremely a deletion that extends into flanking DNA, usually when the template is a wild-type allele present on a homologous chromosome. (Taken from Sentry and Kaiser, 1993).

different result is obtained if the template does not contain the P-element (i.e. is a wild-type allele) at the site corresponding to the P-element donor site. In this case, double stranded break repair restores the donor site to its wild-type pre-insertion sequence; thus appearing as if the P-element had excised precisely from the donor site. The involvement of double-strand gap repair was suggested by the fact that reversion frequencies for heterozygous P-element insertion mutants are 100 times higher than those for homozygous mutants (Engels et al., 1990). Further evidence supporting this hypothesis comes from the work of Gloor et al., 1991. They demonstrated that the introduction of an *in vitro* modified *white* gene into flies containing an active P-element inserted in the *white* gene, resulted in the transfer of mutations to the site of P-element excision. This finding implies that gene conversion (i.e. double stranded break repair) is involved in the repair of excision gaps.

1.8 P-elements as tools for the study of *Drosophila* genetics.

The P-element family of transposable elements has revolutionised *Drosophila* molecular genetics. Not only in terms of providing important insights into the mechanism of eukaryotic transposition, but paving the way for their use as important tools for gene transfer, insertional mutagenesis, enhancer trapping and gene cloning. One important feature of transposable elements, which has been fully exploited, is their ability to induce mutations. The fact that P-element biology is well understood allows us to manipulate the structure and activity of these mutagenic elements.

1.8.1 Germ-line transformation

DNA-mediated germline transformation is an indispensable tool for analysing many problems in *Drosophila* molecular genetics. The molecular analysis of P-elements led to the development of P-elements as vectors for the efficient germ-line transformation of *Drosophila*. The strategy for using P-elements as vectors for gene transfer is based on

imitating the events that take place during a dysgenic cross between P and M strains. Spradling and Rubin (1982); Rubin and Spradling (1982), found that P-elements could be used to transfer cloned genes into *Drosophila* embryos. The procedure was as follows: a plasmid construct bearing a nonautonomous P-element, into which the gene of interest had been inserted, was injected into young M cytotype embryos prior to the cellularisation of the germline. P-element DNA injected into the embryo can become internalised during cellularisation, and can transpose to the genome. Transposase can be supplied in a number of ways. Firstly, one can inject the construct into embryos that express transposase endogenously, for example from a stable genomic transposase source, such as P[*ry*$^+$ Δ2-3](99B). A dominant marker on the P[*ry*$^+$ Δ2-3](99B) chromosome makes it possible to select stable transformed progeny that have lost the transposase source by segregation. Secondly, the construct can be co-injected with a "helper" element, that produces transposase, but is itself incapable of transposition e.g. pπ25.7wc, "wings-clipped" element (Figure 1.7; Karess and Rubin, 1984). Transformed individuals from both methods can then be recovered in the surviving progeny, usually the transposon of interest carries a phenotypic marker to allow identification of transformants. Unfortunately, P-element-mediated transformation has two major drawbacks. Firstly, the frequency with which transformants are recovered appears inversely proportional to transposon length (Spradling, 1986). However, a cosmid vector has been constructed for transducing very large segments (Haenlin et al., 1985) and sequences greater than 40 kb have been successfully transformed, all be it, at significantly low frequencies as compared to smaller vectors. Secondly, the present methods produce insertions located at essentially random chromosomal locations and there can be quantitative position effects on the expression of the transformed gene (Spradling and Rubin, 1983; Hazelrigg et al., 1984; Levis et al., 1985; Laurie-Ahlberg and Stam, 1987). These effects can be alleviated to some extent by including "buffering" sequences, particularly 5' to the insert (Spradling, 1986; Engels, 1989). Researchers advise isolation of several lines containing a number of independent insertions (Spradling, 1986). These can be generated either as primary transformants, or via

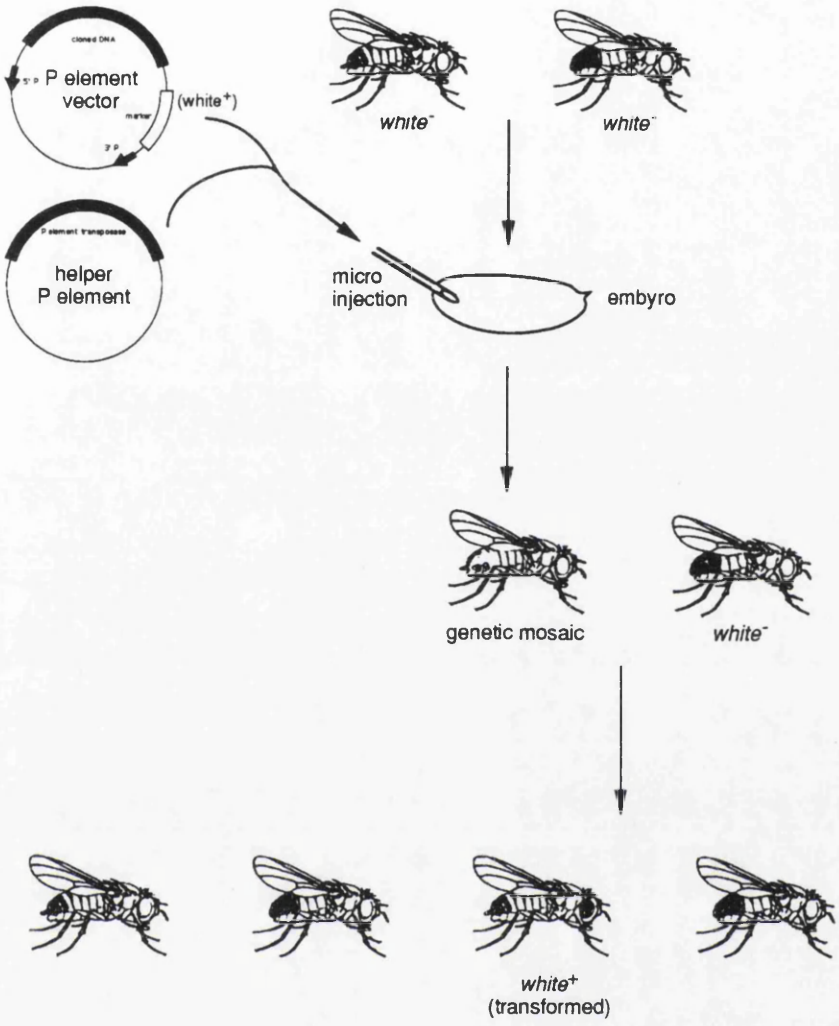


Figure 1.7. Germ-line transformation. A plasmid construct bearing a nonautonomous P-element, into which the gene of interest has been inserted, is injected into young M cytotype embryos prior to the cellularisation of the germline. P-element DNA injected into the embryo can become internalised during cellularisation, and can transpose to the genome. Transposase can be supplied in a number of ways. Firstly, one can inject the construct into embryos that express transposase endogenously, from a stable genomic transposase source, such as P[*ry* + Δ2-3](99B). A dominant marker on the P[*ry* + Δ2-3](99B) chromosome makes it possible to select stable transformed progeny that have lost the transposase source by segregation. Secondly, the construct can be co-injected with a "helper" element, that produces transposase, but is itself incapable of transposition. Transformed individuals from both methods can then be recovered in the surviving progeny, usually the transposon of interest carries a phenotypic marker to allow identification of transformants. (Diagram taken from Sentry and Kaiser, 1993).

remobilisation of a primary insertion line by crossing it to P[*ry*$^+$ Δ2-3](99B) (Robertson et al., 1988).

Germ-line transformation experiments have had a major impact on *Drosophila* molecular genetics for two obvious reasons. Firstly, P-element vectors can be used to transform flies with cloned genes to rescue a mutant phenotype to prove that a DNA fragment carries the corresponding gene, and can be used to identify *cis*-acting regulatory sequences involved in its correct spatial and temporal expression (Bargiello et al., 1984; Bourois and Richards, 1985; Haenlin et al., 1985; Marsh et al., 1985; Fischer and Maniatis, 1986). Secondly, genes manipulated *in vitro* can be reintroduced into the animal and their biological consequences assayed *in vivo.*

1.8.2 Transposon Tagging.

The principle of P-element tagging was first suggested by Bingham et al. (1981), it is useful for loci where the gene product is not known but an efficient mutation screen is available. A typical protocol would be as follows; P-strain males and M-strain females are mated, leading to the induction of P-element transpositions in the germ line of their progeny. These progeny are bred and their offspring are screened or selected for new mutations in the gene of interest (Figure 1.8). In most cases, the new mutations recovered will have resulted from P-element insertions. A library from the resulting mutant strain is then constructed, using a restriction site which does not cut more than once within the P-element sequence. The library is then screened with a P-element probe, hybridising clones will be expected to contain genomic DNA flanking the gene of interest. If the mutant stain has more than one P-element, then the cloned fragments must be screened by *in situ* hybridisation to polytene chromosomes from a strain lacking P-elements. The final step is to screen a wild-type library with the clones to recover the wild-type gene of interest

Using engineered strains (see below; Robertson et al., 1988; Cooley et al., 1988 ; Laski et al., 1986) containing stable transposase-making elements and strains with

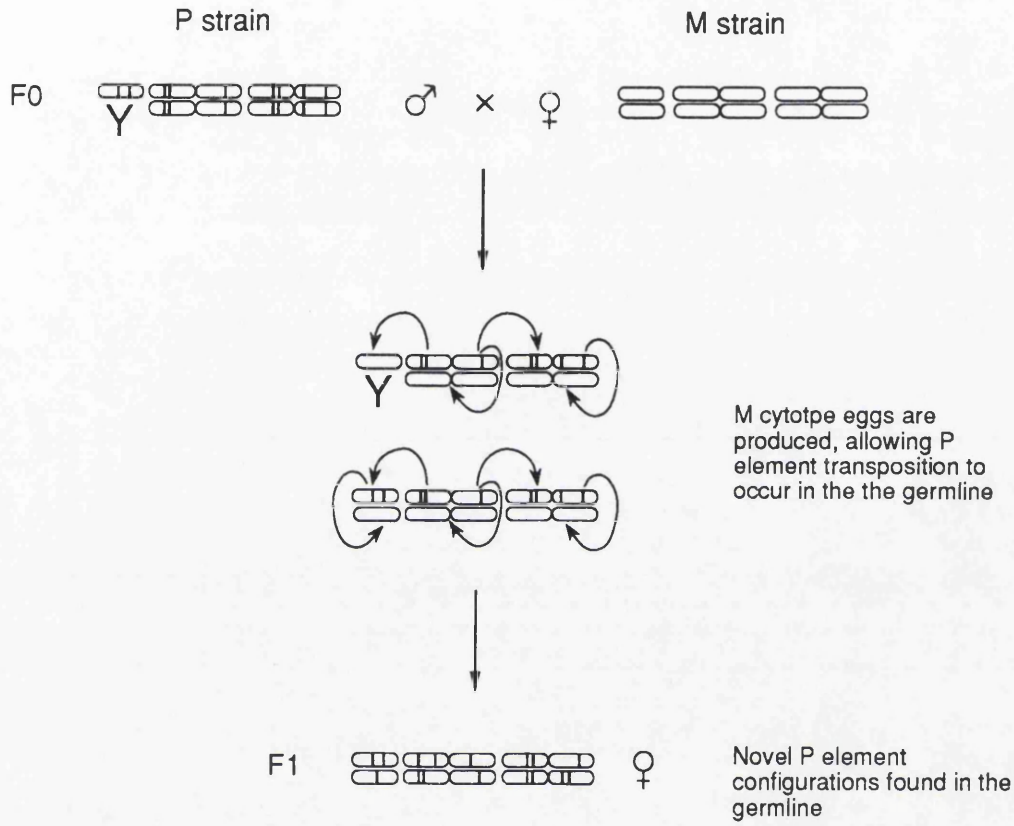


Figure 1.8. Standard mating scheme for P-element mutagenesis. P-strain males and M-strain females are mated, leading to the induction of P-element transpositions in the germ line of their progeny. These progeny are bred and their offspring are screened or selected for new mutations in the gene of interest. (Diagram kindly provided by J. Sentry).

nonautonomous P-elements, crosses can be designed so that the transposase source is present for only a single generation and the resulting insertion is recovered in a transposase free background. It is therefore feasible to recover the mutation in a M strain, and the number of extraneous P-elements in the mutant genome can be minimised. The major strength of transposon tagging as a cloning method is that only the approximate cytogenetic location of the mutation need be known. The only weakness in the method results from the site specificity of P-element insertions (see sections 1.8.3 and 1.8.4) and the type of mutation desired.

In summary, the advantages of P-elements as mutagenic agents are based on their high transposition frequency and the ease with which they can be mobilised in a controlled manner in a dysgenic cross. The P-M system has the potential to induce insertions, deletions, excisions and chromosomal rearrangements. Suitable dysgenic crosses can be set up and subsequently the new mutations can be stabilised or, if appropriate, P-element insertions in or near a gene of interest can be remobilised to recover new alleles of the gene through imprecise excision(see section 1.8.4).

1.8.3 Controlled Mutagenesis

Limitations associated with traditional P-M dysgenic crosses have been overcome by using engineered P-elements, whose activity can be placed under strict experimental control. Robertson et al., 1988, have used a special P-element which produces high levels of transposase, yet is itself remarkably stable. The element P[*ry* $^{+}\Delta$2-3] (99B) has a higher transposase activity than that of an entire P strain, but it rarely undergoes internal deletion, excision or transposition. Due to the removal of its 2-3 intron it makes transposase in both germline and somatic tissues and it carries a dominant marker which allows for selection against the transposase source. Another element called 'Jumpstarter' has been developed by Cooley et al., 1988. This element is not marked and is less stable than Δ2-3.

There are several advantages to using stable transposase-making elements for mutagenesis. Firstly, the stability of Δ2-3 allows its removal by genetic crosses, after which the lack of transposase producing elements ensures that all inserts will be sufficiently stable. Secondly, both Δ2-3 and 'Jumpstarter' can be combined with nonautonomous P-elements. The small but highly mobile nonautonomous P-elements act as "ammunition" to produce insertion mutations. Other elements exist which can be used as ammunition, but these are usually present in much lower copy number in the genome and are more useful for tagging experiments (eg. pUChsneo, Stellar & Pirrotta, 1985). The overall rate of P-element transposition clearly depends on both the number and size of mobilisable elements in the genome and the level of transposase activity. An extremely effective mutagenic strategy (Figure 1.9) involves crossing *Birm-2*, a strain with 17 internally deleted P-elements on each of its second chromosomes, with a strain containing the Δ2-3 element. When ammunition and transposase come together in the germ-line cells of the F1 progeny, transposition of the ammunition elements occurs. Robertson et al (1988) have estimated that approximately ten new insertions per haploid genome occurs in the germ-line cells of a dysgenic individual. It is also desirable to select against the transposase source, to ensure insertion stability, and this is facilitated by a dominant marker linked to the Δ2-3 chromosome. Were P-element insertion completely random, a mutagenised population can be generated of sufficient size that there is a significant probability that any chosen locus will have been a target for insertion(see below). Unfortunately, frequencies of P-element mutagenesis are somewhat locus-dependent (Kidwell, 1986; 1987; Engels, 1989) as the frequency of mutation of different loci varies enormously, from greater than 10^{-3} to less than 10^{-6}. Nonetheless, by applying the cross described above, and given that the estimated *Drosophila* genome size is ~1.65×10^5 kb, the average probability of insertion every 1 kb along the genome would be on the order of 10^{-4} per fly. If the mutagenised population is sufficiently large (10^4 flies), there is a significant probability that any gene could be a target for a P-element insertion.

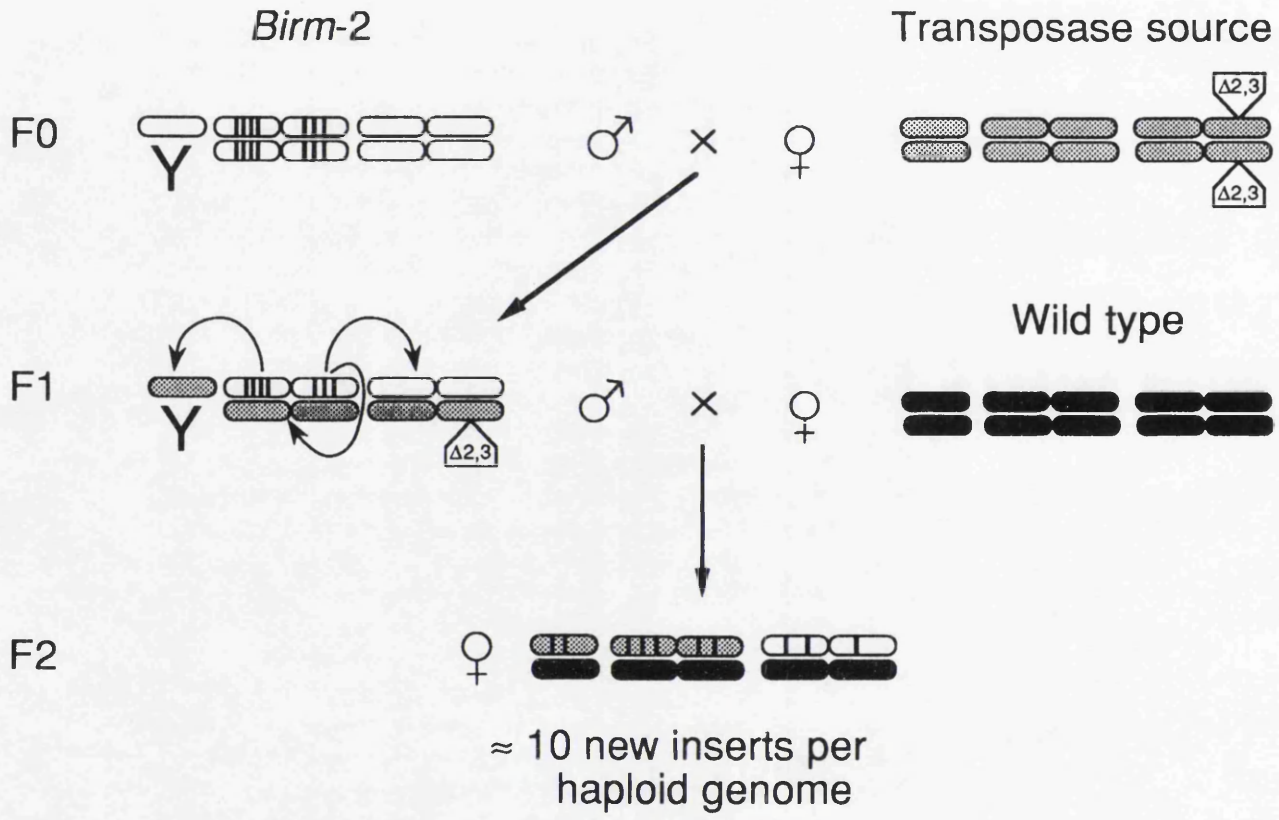


Figure 1.9. A controlled P-element mutagenesis strategy. *Birm-2*, a strain with 17 internally deleted P-elements on each of its second chromosomes, mated with a strain containing the Δ2-3 element. When ammunition and transposase come together in the germ-line cells of the F1 progeny, transposition of the ammunition elements occurs. Robertson et al (1988) have estimated that approximately ten new insertions per haploid genome occurs in the germ-line cells of a dysgenic individual. Selection against the transposase source, ensures insertion stability, and is facilitated by a dominant marker linked to the Δ2-3 chromosome. (Diagram adapted from Cooley et al., 1988, kindly provided by A. Duncanson).

The generation of strains containing only a single marked mutator element, similar to those used for transformation and enhancer trapping (see sections 1.8.1 and 1.8.6), has many advantages as a method of mutagenesis. Phenotypic and molecular analyses of new mutations are greatly simplified. The mutant gene can be mapped, cloned and revertants, including new alleles generated by imprecise excision. Finally, as discussed in section 1.8.5, single-insert lines may have intrinsic long-term value for manipulating the *Drosophila* genome. A drawback with marked elements is their size, they are invariably much larger than unmarked elements, and they subsequently transpose at lower frequencies. In addition, most marked elements are present at only one or few copies per genome. The construction of strains containing many marked elements will help to address this last problem.

1.8.4 Precise and imprecise excision

Remobilisation occurs in the progeny of dysgenic flies carrying the insertion and these events may be precise or imprecise excisions, deletions or chromosomal rearrangements that have their breakpoints at, or in the proximity of, the termini of existing P-element insertions. This procedure has been used in many studies to recover new alleles of a gene. Examples are *rudimentary* (Tsubota and Schedl, 1986), *Sex-lethal* (Salz et al., 1987), and *RpII 215* (Searles et al., 1986). P-elements can undergo three kinds of changes that are generally referred to as excisions;

-Internal excisions : Leave behind some part of the terminal regions of the P-element, range from a few nucleotides to losses of nearly the entire P-element.

-Loss of P-element and flanking DNA : In some cases these events remove sequences on both sides of the element.

-Precise excisions : All of the P-element plus one copy of the 8 bp genomic duplication is lost, thus restoring wild-type sequence.

The data for excision frequencies is plentiful, but caution must be taken with the observed frequencies since it depends critically on which screening procedure was

implemented. Also, as discussed above, there is considerable site-specificity associated with P-element activity and each set of results must be assessed individually.

Many molecular studies of excision events have been conducted for several loci *RpII 215* (Searles et al., 1986), *rudimentary* (Tsubota and Schedl, 1986), *sex-lethal* (Salz et al., 1987), *singed* (Roiha et al., 1988), *white* (Rubin et al., 1982; O'Hare and Rubin, 1983; Levis et al., 1984) and *yellow* (Chia et al., 1986). From all the above studies cited it is possible to draw several conclusions. Firstly, P-element excision requires functional transposase. Secondly, it would appear that the most common event is the internal excision, as opposed to precise excisions or flanking deletions. Whether P-elements are found in 5' control regions, such as *sn, r, RpII 215, Sxl, y,* or found within the coding region, examples being *yellow, white,* it is possible to produce new alleles of these genes by remobilising the P-element and scoring for a change of phenotype.

As discussed in section 1.7, P-element transposition leaves behind a double-stranded break, with a template requirement for repair (Engels, 1990). This finding offers a means by which specific alteration can be made to the *Drosophila* genome. In particular, Gloor et al. (1991) demonstrated that an *in vitro* modified transgene (*white* gene) can serve as the template for repair of the double stranded gap caused by excision of a P-element inserted in the *white* gene (Engels et al., 1990; Gloor et al., 1991; Sentry and Kaiser, 1992). Thus, it may be possible to make specific alterations to any gene that has been "tagged" with a P-element insertion.

1.8.5 Local Jumping

Several observations suggest that when P-elements excise they 'jump' at elevated frequencies into nearby regions of the genome, up to a hundred kilobases or so of the donor site (Tower et al., 1993; Zhang and Spradling, 1993).

Zhang and Spradling (1993), observed that as many as 1% of all progeny, from a dysgenic cross, contained new insertions within 5-200 kb of a donor element. Unusually, the rate of local transposition appears to be much higher in the female germ-

line (1.8%) in contrast to the male germ-line (0.7%). The mechanism(s) causing this effect remains to be elucidated. Nonetheless, this phenomenon can be exploited, given the number of strains throughout laboratories and stock centres, each containing a single marked P-element at a known chromosomal location, it may be possible to local-jump such an element into any gene of interest that is within range.

In fact, this approach has already been used successfully to target a receptor-linked tyrosine phosphatase gene and a synaptotagmin gene (Hamilton et al., 1993; Littleton et al., 1993).

1.8.6 Enhancer Trap Elements

O'Kane and Gehring, (1987) described a powerful method for rapidly identifying and isolating genes involved in *Drosophila* development. The principle of the enhancer trap is as follows. Instead of detecting genes by means of the phenotype caused by a mutation, they are identified by their pattern of expression. One assumes that a developmentally important gene will show a specific temporal and spatial expression pattern related to its function. Enhancer trap elements are modified P elements that can detect transcriptional regulatory elements in the *Drosophila* genome. In these constructs the expression of the *E.coli lac Z* gene, encoding the enzyme ß-galactosidase (ß-gal) is placed under the control of a weak constitutive promoter. These elements are not autonomous, but like the internally deleted P-elements described in section 1.8.1, they can be mobilised in the presence of Δ2-3. In integrated copies of the transposon, the *lac Z* gene acts as a reporter for genomic transcriptional enhancer-like elements or promoters that lie sufficiently close to the transposon to affect this promoter. Thus the pattern of expression of the reporter gene in a line with only a single insertion reflects the temporal and spatial pattern of expression of a flanking gene. The presence of ß-gal activity in any tissue is easily detected by its conversion of the chromogenic substrate X-gal to a blue product. Enhancer trap elements have been modified to include a dominant eye colour gene (w^+) which allows flies with insertions to be recognised, and plasmid sequences that

facilitate the cloning of the flanking genomic DNA (Figure 1.10a; Bellen et al., "The Little Blue Book"). Interestingly, the majority of independent P-*lac Z* insertions lead to non ubiquitous patterns, many of which include or are limited to nervous tissue (O'Kane and Gehring, 1987; Ghysen and O'Kane, 1989; Bellen et al., 1989; Bier et al., 1989; Hartenstein and Jan, 1992). A disadvantage of most "first generation" enhancer trap elements is that they express ß-gal fused to the N-terminal nuclear localisation signal of the P-element transposase. Although nuclear localisation of *lac Z* product makes it easier to visualise and count single cells, cytoplasmic localisation is favourable for the analyses of cells with extensive processes such as neurons (axons and dendrites) and non-neuronal cells with characteristic shapes, such as glia cells (Hartenstein, 1988). Smith and O'Kane (1991), addressed this problem by constructing an enhancer trap element lacking the N-terminal signal, thus allowing cytoplasmic localisation.

A second generation enhancer trap element has now been developed (Figure 1.10b; Brand and Perrimon, 1993; Kaiser, 1993) that provides for expression of a cytoplasmically-localised reporter, and that in addition can be used to target expression of any desired gene product to the marked cells. Instead of ß-gal, the element in question encodes the yeast transcription factor GAL4, which can function in *Drosophila* (Fischer et al., 1988) to turn on expression of transgenes that have been placed under the control of a GAL4-dependent promoter (UAS_G). Crossing a fly having a new GAL4 insertion with a fly containing a UAS_G-*lac Z* construct causes ß-gal to be expressed in a pattern that reflects GAL4 activity.

Enhancer trapping has become a widely used approach for the generation of cell and tissue markers and for the isolation of developmentally regulated genes in *Drosophila.* It is also possible that the insertion of an element may or may not be mutagenic itself (approximately 10% of enhancer trap lines contain insertions that are mutagenic themselves), depending upon its precise location with respect to the gene. Even if insertion of the element does not itself disrupt gene function, then the element can be remobilised to generate imprecise excisions. This approach is facilitated by a dominant eye-colour marker carried by the enhancer trap element, whose loss can be easily scored

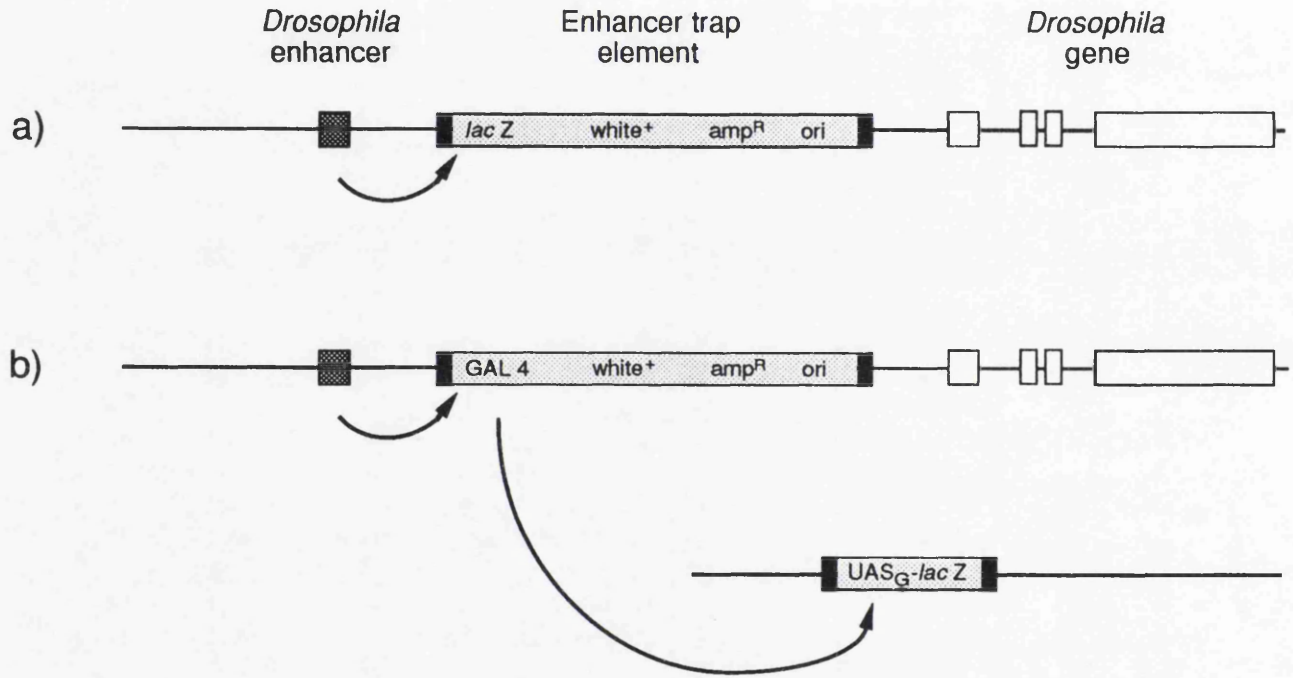


Figure 1.10. Enhancer trapping.

a) First generation enhancer trap element. After integration into the genome, the reporter may be influenced by nearby genomic enhancers, leading to developmental regulation of β-galactosidase expression. Enhancer trap elements have a dominant eye colour gene (w^+) which allows flies with insertions to be recognised, and plasmid sequences that facilitate the cloning of the flanking genomic DNA .

b) A GAL4 enhancer trap element. As above, except that GAL4 expression is influenced by a nearby enhancer. GAL4 activates transcription from a second reporter gene linked to the GAL4 responsive promoter, UAS_G. (Diagram taken from Sentry et al., 1993a).

in an appropriate excision screen (see section 1.8.4). Furthermore, the GAL4 system will allow the expression of any gene in marked cells under the control of UAS_G. For example, dominant negative mutations of a gene, peptide inhibitors of specific signal transduction pathways and antisense constructs to knock out endogenous gene expression could be used. One can then assay the consequences of ectopically expressing that construct in the cells in which the enhancer trap is active. One of the most powerful applications of this technique will be the ability to ablate specific cells by expressing cell-autonomous toxin genes such as ricin and diphtheria under the control of a UAS_G. In particular the use of temperature-sensitive toxins may allow the control of temporal aspects of ablation (Bellen et al., 1992; Moffat et al., 1992; O'Kane and Moffat, 1992; Sentry et al., 1993; Kaiser, 1993).

The major goal of my Thesis is to develop a reverse genetics approach, enabling the generation of mutants corresponding to any chosen gene, in particular, genes implicated in learning and memory. This approach exploits the mutagenic properties of the P-element (see above) and the polymerase chain reaction (PCR) (see chapter 3).

Chapter 2

Materials and Methods

2.1 *DROSOPHILA*

2.1.1 Stocks

A description of the *Drosophila* stocks used in this work can be found in Table 2.1. A description of all mutations used can be found in Lindsley and Zimm, 1992.

2.1.2 Culture

All flies were routinely raised on Glasgow medium. Culture temperatures were 25°C, unless otherwise stated. A grape juice agarose egg medium was used to rear flies used in sib-selection experiments. Third instar larvae, used for *in situ* hybridisation studies to polytene chromosomes, were reared on a rich medium. Finally, strains used in behavioural assays were maintained at 25°C on a 16/8 hr dark/light cycle on high learning medium.

Glasgow medium: 10g agar, 15g sucrose, 30g glucose, 35g dried yeast, 15g maize meal, 10g wheat germ, 30g treacle, 10g Soya flour per litre of water.

Grape juice agarose medium: 19.8g agarose, 52.2g glucose, 26g sucrose, 7g dried yeast, 9% (v/v) red grape juice (Safeway) per litre of water.

Rich medium: 100g glucose, 100g dried yeast, 20g agar per litre of water.

High learning medium: 8.4g agar, 31.9g dried yeast, 94.2g glucose, 8.7g potassium tartrate, 7g calcium chloride, 71.6g maize meal per litre of water (Tully and Quinn, 1985).

Strain/Genotype	*Reference*
Oregon R	Lindsley and Zimm, 1992
Canton S (High Learning Index) From T.Tully.	Lindsley and Zimm, 1992
M56i	Schalet and Lefevre, 1973
Birm-2 ; ry^{506}	Engels et al., 1987
w ; *Sb P* [ry^{+} Δ2-3](99B)/TM6	Robertson et al., 1988
w ; *Dr P* [ry^{+} Δ2-3](99B)/TM6B, *Tb, Hu, e*	Robertson et al., 1988
w ; TM3, *Sb* , *Ser* , *e* /TM6B, *Tb, Hu, e*	Lindsley and Zimm, 1992
C(1)M3/FM6/Y; *bw, st*	Lindsley and Zimm, 1992
Harwich	Kidwell, 1986
CFL3	Roiha et al., 1988
CFL5	Roiha et al., 1988
Cy O/*Sp*	Lindsley and Zimm, 1992

Table 2.1. *Drosophila* stocks used in this study.

For all fly media, the water was heated to boiling to dissolve the agar or agarose, the remaining reagents added and dissolved and the media allowed to cool to 60°C. Once cooled to 60°C, Nipagen M (4-hydroxybenzoic acid methylester, 10% (w/v) in absolute ethanol) was added to a final concentration of 0.1% (v/v) to inhibit fungal growth in *Drosophila* cultures.

2.1.3 Behavioural assays

Associative learning

Flies were trained with the classical conditioning procedure of Tully and Quinn (1985). To begin training, a group of roughly 100 tester or control flies, 1-4 days old, are trapped inside a chamber which contains an electrifiable grid on 90% of its inner surface. Flies are exposed sequentially to two odours, 3-octanol (OCT; ICN-K&K Labs) and 4-methylcyclohexanol (MCH; ICN-K&K Labs), carried through the training chamber on a current of air (750ml/min). Flies are first exposed for 60 sec to the conditioned stimulus (CS^+; either OCT or MCH), during which time they receive the unconditioned stimulus (US), twelve 1.25 sec pulses of 60V DC electric shock at 5 sec interpulse intervals. After presentation of the CS^+, the chamber is flushed with fresh air for 30 sec. Then, flies are exposed for 60 sec to a second, control stimulus (CS^-; either MCH or OCT), which is not paired with electric shock. After presentation of the CS^-, the chamber again is flushed with fresh air for 30 sec. After training, flies are tapped gently from the training chamber into an elevator-like compartment that transports them to the choice point of the T-maze, where relative (conditioned) odour avoidance responses are assayed by exposing the flies to two converging currents of air (1500ml/min at the choice point) one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies are allowed to choose between the CS^+ and CS^- for 120 sec, at which time they are trapped inside their respective arms of the T-maze (by sliding the elevator out of register), anaesthetised and counted. Flies that chose to avoid the CS^+ ran into the arm containing

the CS^-, while flies that chose to avoid the CS^- ran into the arm containing the CS^+. A small percentage of flies (<5%) usually remained at the choice point . Relative concentrations of OCT and MCH were adjusted so that naive flies distributed themselves 50:50 in the T-maze. All training and testing was done at 25°C and 70% relative humidity in dim red light (650nm). Two groups of flies were conditioned in one complete experiment. The CS^+ was OCT and the CS^- was MCH for one group; the CS^+ was MCH and the CS^- was OCT for the second group. For each reciprocal group, the "probability correct " (COR) was calculated as the number of flies avoiding the CS^+ divided by the total number of flies in the T-maze arms:

$$COR = [CS^+]/([CS^+] + [CS^-])$$

These two COR values then were averaged, and that average was normalised to produce one performance index (PI):

$$PI = \frac{[(COR_{OCT} + COR_{MCH})/2] - 0.5}{0.5} \times 100$$

$$= [(COR_{OCT} + COR_{MCH}) - 1] \times 100$$

This index creates scores ranging from 0 (no learning) to 100 (perfect learning). Calculation of a PI in this manner is algebraically equivalent to the learning index (I) of Tully and Quinn (1985), except that the number of flies left at the choice point is included in the calculation of the latter. Typically about 5% of the total number of flies remain at the choice point. Nonetheless, the PI is a measure of associative learning unaltered by any nonassociative changes in odour avoidance that may occur during classical conditioning.

Memory retention

Groups of flies were trained as above, then removed from the training chamber and stored in the dark for a period of 15, 30, 60 or 180 min in test tubes containing food medium. Seventy seconds before the usual 2 min test trial, flies were transferred to the choice point of the T-maze. For retention intervals of 0 min, flies were transferred to the T-maze 1.5 min after training (3.5 min after the CS^+ presentation). An equal number of performance indices was then collected for each retention interval.

Olfactory acuity

Absolute odour avoidance responses were quantified by exposing a group of roughly 100 tester or control flies to each odour (either OCT or MCH) *vs.* air in the T-maze. After 2 min, the number of flies in each arm of the T-maze was counted, and a performance index was calculated for each odour individually:

$$PI_{OCT} = \frac{CO_{OCT} - 0.5}{0.5} \times 100$$

$$= [2CO_{OCT} - 1] \times 100$$

$$PI_{MCH} = \frac{CO_{MCH} - 0.5}{0.5} \times 100$$

$$= [2CO_{MCH} - 1] \times 100$$

The odour and air were presented in the left and right arms of the T-maze, respectively, for half of the experiments; the reciprocal arrangement was used for the other half. In this way, any environmental cues that might have produced small "side biases" were eliminated from the mean PI for the group.

Shock reactivity

The ability to sense electric shock and to escape from it is quantified by inserting electrifiable grids into both arms of the T-maze. A group of roughly 100 tester or control flies were aspirated into one arm and exposed to one 1.2 sec shock pulse before the centre compartment is opened, thereby allowing flies to escape into the opposite, unshocked arm of the T-maze. Thereafter, shock pulses continued to be delivered every 5 sec, and flies were allowed to escape into the opposite T-maze arm. After 60 sec, the centre compartment was closed, trapping flies in their respective arms. Flies in the unshocked T-maze arm (UA) were considered to have escaped electric shock. For each group the COR was calculated as the number of flies avoiding the shocked arm (SA) divided by the total number of flies in the T-maze arms. This COR value was then normalised to produce a performance avoidance index:

$$COR = [SA]/([SA] + [UA])$$

$$PA = \frac{COR - 0.5}{0.5} \times 100$$

$$= [2COR-1] \times 100$$

This index creates scores ranging from 0 (no avoidance) to 100 (complete avoidance). During this test, amenotactic cues were provided by drawing air through the arms of the T-maze and out the centre compartment (1500ml/min). Again to eliminate any side bias in mean scores, equal numbers of groups of flies were shocked in either the left or right arm of the T-maze.

Locomotor activity

Locomotor activity was assessed by using the countercurrent phototaxis apparatus (Benzer, 1967; without light). Approximately 100 flies, of the appropriate genotype, were loaded into a start tube which was aligned with a second, distal tube. The flies were tapped to the bottom of the start tube, which then was laid horizontally on a table. The flies were allowed to distribute themselves between the start tube and the distal tube(s) for 30 sec. The two tubes were then shifted out of alignment, separating those flies that ran into the distal tubes from those remaining in the start tube. The flies in the distal tube were tapped into a second start tube, and then both groups again were allowed to run into distal tubes for 30 sec . This procedure was done five times, separating the flies into six groups based on the number of times a fly ran into a distal tube. The most active flies ran into distal tubes five times and received a score of five; the least active flies remained in the original start tube, receiving a score of zero.

Habituation of the jump reflex to olfactory cues

Males were collected under CO_2 anaesthesia on the day of eclosion and were then stored in food vials (~10 males/vial) at 25°C for 2 days. Locomotor activity in 2 day old males was measured the day of the habituation experiment (see above). Only individuals with an activity score of zero were discarded. Single males were placed in chambers consisting of a Lucite base and a plastic test tube (Falcon). A vacuum source was connected to the base of the chamber, drawing air (1000ml/min) through distilled water, in a 3 mm hole at the top of the test tube, through the chamber and out nylon mesh-covered holes in the base. At regular intervals, a computer controlled (Commodore, Program by Dr T.Tully), 3-way solenoid valve switched from the flow of "Fresh" air to one that was bubbled first through distilled water and then through 10% benzaldehyde (Fluka) in heavy mineral oil (Fisher). Usually eight or sixteen flies were tested in one experiment. Each fly was habituated by exposing it repeatedly to 10% benzaldehyde for 4 sec at 1 min intertrial

intervals. Each fly was judged to have habituated when it failed to jump during the 4 sec odour presentation in four consecutive trials (4 no-jumps). A fly's habituation score was the number of trials needed to obtain a block of 4 no-jumps (trials to criterion). Spontaneous recovery was measured at various time intervals (2, 5, 15, 20 and 30 minutes) after a fly reached criterion by exposing it one more time to a 4 sec pulse of benzaldehyde. The percentage of flies that jumped during this test trial reflected the average amount of spontaneous recovery for each genotype. The apparatus has been automated (Boynton and Tully, 1992) and usually 8 or 16 flies were habituated in one experiment. In all cases, equal numbers of each genotype were tested. These experiments were carried out at room temperature in ambient relative humidity between 8 a.m. and 12 noon

Statistics

Since a PI itself is an average of percentages, a sample of performance indices is distributed normally. Therefore, statistical analyses in this study were based on analyses of variance (ANOVA) with unplanned pairwise comparisons among group means (Sokal and Rohlf, 1969). Computations were done on a VME mainframe computer with MINITAB statistical software (Minitab project, Penn State University).

2.1.4 Nucleic acid isolation

DNA isolation

Egg DNA isolation

Eggs were washed in tap water and homogenised in 500µl or more of 10mM Tris-HCl, pH 7.4, 10mM EDTA, 60mM NaCl, 0.15mM spermine, 0.15mM spermidine, 0.5% Triton X-100®. The homogenate was extracted once with an equal volume of phenol/$CHCl_3$ (1:1 mixture). The DNA was precipitated by the addition of 0.1vol of 3M NaOAc, pH 5.0, and 2.5 vol of EtOH, and storage at -20°C for 1-2hr. The DNA was pelleted (12,000g, 5min) at 4°C. The pellet was washed with 70% EtOH, dried and resuspended in 100-1000µl of TE (1mM EDTA, 10mM Tris-HCl, pH 8.0) containing 10µg/ml of RNase A (From a 10mg/ml stock solution in 10mM Tris-HCl, pH 7.5, 12mM NaCl, boiled for 15 min to destroy any DNase activity). From a cage with a 1000 females laying eggs, approximately 1µg of egg DNA was obtained per female.

Rapid single fly DNA isolation for PCR

Single-fly DNA was prepared by the method of Gloor and Engels (pers. comm.). Single flies were homogenised in a 1.5ml Eppendorf microcentrifuge tube with an Eppendorf micropestle in 50µl of homogenisation buffer (10mM Tris-HCl, pH 8.3, 1mM EDTA, 25mM NaCl, 200µg/ml Proteinase K, from a 20mg/ml stock solution in sterile distilled water). The homogenate was incubated for 30 min at 37°C, then heated to 95°C for 2 min, and 1/25th used directly in a PCR reaction.

General single fly DNA isolation

Single-fly DNA was prepared by modifications to the procedure of Ashburner (1989). A single-fly was homogenised in a 1.5ml Eppendorf microcentrifuge tube with an Eppendorf micropestle in 50µl of homogenisation buffer (10mM Tris-HCl, pH 7.5, 60mM NaCl, 10mM EDTA, 0.15mM spermine, 0.15mM spermidine, 5% (w/v) sucrose) and then 50µl of lysis buffer (0.3M Tris-HCl, pH 9.0, 100mM EDTA, 5% (w/v) sucrose, 0.625% SDS (w/v), 1% DEPC (v/v)) was added. The homogenate was incubated for 15 min at 70°C, then cooled to room temperature and 0.15 vol of 8M potassium acetate added. The homogenate was incubated on ice for 30 min and then centrifuged (12,000g, 10min) at 4°C. The supernatant was carefully removed to a fresh tube and extracted once with an equal volume of phenol/$CHCl_3$ (1:1 mixture).

The DNA was precipitated by the addition of 2 vol of EtOH, incubated at room temperature for 5 min and pelleted (12,000g, 5min). The pellet was washed with 70% EtOH, dried and resuspended in 20µl of TE. RNase A (From a 10mg/ml stock solution in 10mM Tris-HCl, pH 7.5, 12mM NaCl, boiled for 15 min to destroy any DNase activity) was added to a final concentration of 50µg/ml. Yields of DNA were generally 1µg/fly.

Large scale DNA isolation

Drosophila high molecular weight chromosomal DNA was prepared by modifications to the procedure of Levis et al., 1982. One gram of flies was ground in liquid N_2 with a precooled mortar and pestle and homogenised with 9ml of ice cold homogenisation buffer (30mM Tris-HCl, pH 8.0, 10mM EDTA, 100mM NaCl, 10mM ß-mercaptoethanol, 0.5% (v/v) Triton X-100®) in a Wheaton 15ml homogeniser (A pestle). The solution was decanted through nylon gauze into a sterile 30ml corex tube on ice and the nuclei pelleted (4,000g, 10min) at 4°C. The supernatant was carefully removed and the nuclei resuspended in 1ml of ice cold homogenisation buffer (Without

0.5% Triton X-100®) by gentle pipetting, 5ml of nuclear lysis buffer (100mM Tris-HCl, pH 8.0, 100mM EDTA, 100mM NaCl, 0.5mg/ml Proteinase K) was added. The nuclei were lysed by addition of 0.2ml of 30% (w/v) sarkosyl followed by gentle swirling of the tube. The lysate was incubated at 37°C for 12 hr and then 1.25g CsCl per gram of lysate was added. The lysate was loaded into a 16 x 76mm Beckman polyallomer Quick-Seal™ centrifuge tube and topped up with 1.25g/ml CsCl/water and sealed. This was then spun for up to 60 hr at 45,000rpm at 25°C, in a Beckman Ti70 rotor. After centrifugation the tube was punctured with a 18G needle at the top and an 18G needle near the bottom. The gradient was dripped through the bottom needle (controlling the flow with the top needle at the top) and collected into 0.5ml fractions. The fractions with the most DNA were determined by spotting onto Ethidium bromide plates (1% Agarose, 0.5µg/ml of EtBr). The fractions containing DNA were pooled and dialysed extensively with TE (4 litres of TE with one change) and stored at 4°C. Yields of DNA were generally 100-200µg/g of starting material.

RNA and poly (A)$^+$ RNA isolation

Adult body and head was collected as previously described (Levy and Manning, 1981). RNA was isolated by a modification of Chomzynski and Sacchi (1987) that is described in the laboratory manual of K. Stanley (EMBL Heidelberg), followed by batch purification of poly(A)$^+$ mRNA using oligo(dT)-cellulose. Briefly, one gram of tissue was homogenised at full speed with a Kinematica Polytron® tissue homogeniser for 2-3 min in 10ml of denaturing solution (4M guanidinium isothiocyanate, 0.1M Tris-HCl, pH 8.0, 0.1M ß-mercaptoethanol, 0.1% (v/v) antifoam-A). To the homogenate the following were added sequentially, 0.1 vol 2M NaOAc pH4, an equal volume of phenol and 0.2 vol $CHCl_3$. The mixture was vortexed, left to stand on ice for 15-30 min and centrifuged (12,000g, 10 min at room temperature). The aqueous phase was removed to a fresh tube, 1 vol of isopropanol added and the mixture left at -20°C for 1hr to allow the RNA to precipitate. The precipitate was pelleted (12,000g, 10 min at room

temperature) and resuspended in 5ml of denaturing solution. The RNA was again precipitated as described above, washed in 70% EtOH, and finally resuspended in 1ml of oligo(dT)-cellulose binding buffer (0.5M NaCl, 10mM Tris-HCl, pH 7.5, 1mM EDTA, 0.1% (w/v) sarkosyl). Oligo(dT)-cellulose (50mg/ g tissue) was hydrated overnight at 4°C in H_2O, treated for 15 min at room temperature with 0.1M NaOH and washed (5 x 10ml sterile H_2O, 5 x 10ml binding buffer). The RNA was heated to 68°C for 5 min, chilled on ice, thoroughly mixed with the oligo(dT)-cellulose in a 15ml Falcon tube and incubated at room temperature for 30 min with gentle agitation. The oligo(dT)-cellulose was pelleted by centrifugation (15,000g, 10 min at room temperature) and washed 5 times with 10ml of binding buffer for each wash. The poly(A)$^+$ mRNA was eluted by resuspending the resin in 0.5ml of sterile H_2O and incubating at 58°C for 15-30 min with occasional shaking. The oligo(dT)-cellulose was pelleted (3,000g, 10 min at room temperature) and the supernatant transferred to a 1.5ml microcentrifuge tube. The RNA was precipitated by the addition of 50µl 3M NaOAc, pH 5.0, 1ml EtOH, stored at -20°C for 1-12 hr and collected by centrifugation in a microcentrifuge for 30 min at 4°C. The RNA was resuspended in 100µl of sterile H_2O and stored at -70°C Yields were generally 50µg of poly(A)$^+$ mRNA/g of tissue.

2.1.5 *In situ* hybridisation

Tissue sections

Wild type flies were mounted side by side in a fly collar, soaked in O.C.T. compound (Lab-Tek Division) for 30 min, frozen at -18°C, cut as 7 µm sections in a cryostat (Anglia Scientific, Cryotome 620), placed on gelatinised slides, and postfixed in freshly made PLP fixative (4% (w/v) paraformaldehyde, 0.01M sodium metaperiodate, 0.075 M lysine, 0.044M NaCl, 0.037M phosphate buffer, pH 7.2) for 10 min. After three washes in PBS (140mM NaCl, 50mM phosphate buffer, pH 7.4) , they were treated with 0.2N HCl for 20 min, washed in 2X SSPE (0.36M NaCl, 0.02M NaH_2PO_4, 2mM EDTA,

pH 7.8) for 30 min at 65°C, treated with 350 µg/ml of auto-digested pronase in 50mM Tris-HCl and 5mM EDTA, pH 8.0, incubated in 2 mg/ml glycine in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and acetylated with 0.25% (v/v) acetic anhydride and 0.1M triethanolamine in PBS for 8 min. The sections were incubated with 150-200 µl of prehybridisation solution at 42°C for 2-3 hr. They were then incubated with single-stranded DNA probes at 42°C. After overnight incubation, the sections were washed extensively, three times (15 min each) in 2 X SSPE, once in 1 X SSPE, and once in 0.5 X SSPE at room temperature. A final wash was performed in low salt buffer (2mM NaPPi, 1mM $NaPO_4$, 1mM EDTA, pH 7.2) at 42°C. For immunological detection of the hybridised probes, the sections were incubated with 200 µl of 2% (v/v) sheep serum (Gift from S.A.P.U. Law Hospital, Carluke, Scotland) in buffer I (100mM Tris-HCl, pH 7.5, 150mM NaCl and 0.3% (v/v) Triton X-100®) for 60 min. The sections were incubated with 150-200 µl of anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted at 1:500 in buffer I without 0.3% (v/v) Triton X-100® for 2-3 hr. After extensive washes in 100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM $MgCl_2$, the sections were placed with 200 µl of diluted chromogenic substrate solutions (NTB and BCIP, X-phosphate) following the manufacturer's instructions (Boehringer Mannheim), incubated in the dark at room temperature for 2-6 hr. The reaction was stopped by washing in 10mM Tris-HCl, 1mM EDTA, pH 8.0 for 10 min, washed in H_2O, dehydrated, and mounted with glycerol gelatin (Sigma).

Polytene chromosomes

Isolation and treatment of *Drosophila* salivary glands was performed as described by Pardue, 1986). The probe was a pBluescript recombinant labelled with Bio-16-dUTP (Boehringer Mannheim) by nick translation (see section 2.3.5). Hybrids were detected with Streptavidin-Biotinylated Horseradish Peroxidase complex and diaminobenzidine/

H_2O_2 as described by Pardue (1986). The slides were stained with Giemsa and the coverslips were mounted with D.P.X. (BDH).

Tissue sections and polytene chromosome preparations were photographed using a Leitz Vario-orthomat camera system and Kodak Echtachrome 160 Tungsten film.

2.2 BACTERIAL PROTOCOLS

2.2.1 Strains and plasmids

The only bacterial strain used in this study was XL1-Blue (Bullock, 1987; Stratagene), a derivative of *Escherichia coli* K-12. Its genotype is given below.

Strain	*Genotype*	*Reference*
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44 relA1, lac,* [F' *proAB, lacI*^{q}*ZΔM15,* Tn*10* (tet^r)].	Bullock (1987)

Plasmids used in this study, other than those whose construction is described elsewhere in this thesis, are listed below.

Plasmid	*Description*	*Source/Reference*
pSn9	*Eco*RI fragment of the *D. melanogaster* singed gene in pBR322	Roiha et al., 1988 Gift from K. O'Hare.
pBluescript®II KS+/-		Mead et al., 1985 Stratagene USA
pBluescript®II SK+/-		Mead et al., 1985 Stratagene USA

Plasmid	*Description*	*Source/Reference (cont..)*
pcD6BO RXba	*Eco*RI -*Xba*I fragment of type Ia RI cDNA in pBluescript®II KS+.	Kalderon & Rubin (1988) Gift from D. Kalderon.
pBR*rp49*	*Eco*RI -*Hin*dIII fragment of the *Drosophila* ribosomal protein 49 gene gene in pBR322.	O'Connell & Rosbash (1984)
pST41	*Eco*RI -*Hin*dIII fragment of *nina E* gene, major opsin of *D. melanogaster* in pBluescript®II SK-	Gift from S.Tomlinson
pπ 25.7 WC	P-element sequences from position 1 to 2885 inserted between the *Hin*dIII and *Sal*I sites of pBR322.	Gift from K. O'Hare
λgS8	EMBL3 derivative containing a fragment of the *Drosophila* RI genomic DNA.	Russell and Kaiser, 1993

2.2.2 Culture medium

L-Broth: 10g Bacto-tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, per litre of water and adjusted to pH 7.0 with NaOH.

L-Agar: As above with the addition of Bacto-agar (Difco) to 1.5% (w/v).

All culture media was sterilised by autoclaving at 120°C for 15 min.

2.2.3 Antibiotics and indicators

When necessary either ampicillin, at a final concentration of 50µg/ml (50mg/ml stock solution in sterile distilled water), or tetracycline, at a final concentration of 12.5µg/ml (12.5mg/ml stock solution in absolute ethanol) were added to broth or agar. 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and Isopropyl-ß-D-thiogalactopyranoside (IPTG) were added to molten agar (50°C) in order to detect recombinant clones. X-gal was dissolved in dimethylformamide, and IPTG in sterile distilled water. Both were stored at -20°C as 20mg/ml solutions, and used at a final concentration of 20µg/ml.

2.2.4 Transformation of *E. coli*

Preparation of competent cells

20ml of L-broth was inoculated with 0.4ml of an overnight culture of XL1-Blue , and grown with aeration at 37°C for 90 min, or until cells had entered the logarithmic growth phase(OD_{600} = 0.4). The cells were then pelleted at 4000g for 5 min at 4°C in a bench-top centrifuge, the supernatant removed, and the resulting pellet resuspended in 10ml ice-cold 50mM $CaCl_2$ solution. After a 20 min incubation on ice the cells were repelleted as above, and then suspended in 2ml ice-cold 50mM $CaCl_2$ solution. Competent cells were either used fresh, or after 24 hr at 4°C.

Transformation

50-150ng of DNA in a volume up to 10µl was added to 200µl of competent cells and left on ice for 45 min, before the mixture was subjected to a heat-shock at 42°C for 2 min, cooled for 1 min on ice, and plated onto L-agar plates containing the appropriate antibiotics and indicators, and incubated overnight at 37°C to select for transformants.

2.2.5 Preparation of plasmid DNA

Large scale plasmid isolation was carried out by the alkaline-lysis method of Birnboim and Doly, 1979, as described in Sambrook et al., (1989). Small scale plasmid isolation was carried out with the Magic™ DNA purification system (Promega), using the conditions recommended by the manufacturers.

2.3 GENERAL MOLECULAR BIOLOGY PROTOCOLS

2.3.1 Quantification of nucleic acids.

For quantitating the amount of DNA or RNA in a sample, readings were taken at wavelengths of 260nm or 280nm. An OD_{260} =1 corresponds to ~50µg/ml for double-stranded DNA, ~40µg/ml for RNA and ~33µg/ml for oligonucleotides. When samples had limiting concentrations of DNA (< 250ng/ml), the quantity of DNA was estimated by spotting the sample and known standards onto the surface of a 1% (w/v) agarose gel containing EtBr (0.5µg/ml). The gel was photographed using short-wavelength UV illumination (254nm) and the concentration of the DNA sample was estimated by comparing the intensity of fluorescence in the sample with those of known DNA concentration standards.

2.3.2 Oligonucleotide synthesis

Oligonucleotides were synthesised by the solid state method on an Applied Biosystems Inc. PCR-MATE 391 DNA Synthesiser, employing phosphoramidite chemistry. After ammonium hydroxide cleavage and deprotection, oligonucleotides were evaporated to dryness under vacuum and resuspended in water. Typically primers were 22-31nt in length having 50 to 60% G+C composition. Wherever possible T_ms for a given primer pair were balanced, any possible secondary structure and complementarity to each other

was minimised. T_ms were estimated by the calculation of Thein and Wallace, 1986. 2°C for A or T and 4°C for G or C. A description of the oligonucleotides used in this study can be found in Table 2.2.

2.3.3 PCR

PCR conditions

Generally unless otherwise stated PCR reactions were carried out on 100-200ng of template DNA in 20µl of 50mM KCl, 10mM Tris-HCl (pH 8.3 at room temperature), 1.5mM $MgCl_2$, 0.01% (w/v) Triton X-100®, 200µm dATP, 200µm dCTP, 200µm dGTP, 200µm dTTP, the primers, each at between 1µm-0.33µm and 1 unit of *Taq* polymerase (Promega). Samples were overlain with an equal vol of mineral oil (Sigma) and PCRs were performed in a Hybaid Thermal Reactor (Hybaid) with an initial denaturation step of 3 min at 94°C, followed by a three step routine that consisted of a 1 min annealing at 55-60°C, extension at 72°C for 3 min and denaturation at 94°C for 1 min. A total of 30 cycles were carried out, followed by a return to 55-60°C for 5 min and a further 20 min extension step at 72°C, and then a return to room temperature. PCR products destined for cloning were purified with the Magic™ PCR purification system (Promega), using the conditions recommended by the manufacturers.

Cloning PCR products

PCR products were cloned into pBluescript®II by exploiting the terminal transferase activity of *Taq* polymerase (Clark, 1988). The vector was digested with *Eco*RV and incubated with *Taq* polymerase (1U/µg plasmid/20µl vol) using standard buffer conditions (50mM KCl, 10mM Tris-HCl; pH 8.3, 1.5mM $MgCl_2$, 0.01% (w/v) Triton X-100®) in the presence of 2mM dTTP for 2hr at 70°C. The DNA was then purified using the Magic™ DNA purification system (Promega), using the conditions

Primer Type	*Primer*	*Orientation*	*Sequence (5'-3')*	*T_mS*	*Reference*
31 bp terminal inverted repeat of the P-element	P_{31}	+/-	CGACGGGACCACCTTATGTTATTTCATCATG	90°C	O'Hare and Rubin, 1983
31 bp terminal inverted repeat of the P-element (inverse)	P_{31Inv}	+/-	CCATGATGAAATAACATAAGGTGGTCCCG	90°C	O'Hare and Rubin, 1983
Internal P-element primer	P_R	+/-	AGCATACGTTAAGTGGATGTCTC	66°C	O'Hare and Rubin, 1983
Internal P-element primer	P_L	-/+	GTGTATACTTCGGTAAGCTTCGG	68°C	O'Hare and Rubin, 1983
P-element sequencing primer	$P_{233-247}$	+	ATTGTGGGAGCAGAG	44°C	O'Hare et al., 1992
P-element sequencing primer	$P_{2397-2412}$	+	GACATTTACATACGTC	52°C	O'Hare et al., 1992
Gene-specific primer, *singed* gene	SnA	-	GTCTGTCGTCACACCCTTCACTTCGCC	86°C	O'Hare pers. comm.
Gene-specific primer, *singed* gene	SnB	+	GCTGCCCTTCTAATCCTCGCGTCC	78°C	O'Hare pers. comm.
Gene-specific primer, RI gene; exon I	77F1	-	GATGCAGTCCTTGAGGACTCGC	70°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; exon I	77Fex1	-	TATTGTTTCGCTGGCAAATCGG	64°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; exon I	77F2	+	AGAATCCCGTGCAGTTCCTGCG	70°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; exon I	77F3	+	ACCCACCTCCGTTGATTGGCTC	70°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; exon I	77Fdown	+	AAACAACAACGTTTGACGTGCG	64°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; exon I	77Fup	-	TGGTCACGTTCATATCGATAGG	64°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; common region	77FC1	+	TTGCCAAGAACGTCCTGTTCGC	68°C	Kalderon and Rubin, 1988
Gene-specific primer, RI gene; common region	77FC2	-	CGCCAAAGTAATCGCTGCTACC	68°C	Kalderon and Rubin, 1988
Gene-specific primer, ductin gene	JP1	+	GCTTTTAACGAGCAGCCAGCAG	68°C	Meagher pers. comm.
Gene-specific primer, ductin gene	JP2	-	TTCAAGTTGTCGCAGCAGCATG	66°C	Meagher pers. comm.
Gene-specific primer, ductin gene	JP3	-	GTCGCTGCTCACTTCAGAAGAC	68°C	Meagher et al., 1990
Gene-specific primer, ductin gene	JP4	+	TTGACGTCCATCAGGTGGCAGC	63°C	Meagher pers. comm.

+ Forward Primer
- Reverse Primer
+/- Forward or reverse depending on orientation of P-element

Table 2.2. P-element and gene-specific oligonucleotide primers used in this study.

recommended by the manufacturers. DNA ligations were carried out in a final volume of 20µl, using 200ng of T-tailed pBluescript®II, with a 3-4 fold excess of insert DNA to vector. 4µl of 5 X Ligation buffer (250mM Tris-HCl , pH 7.6, 50mM $MgCl_2$, 25% (w/v) PEG 8000, 5mM DTT, 5mM ATP) was added to the DNA, and the volume made up to 20µl with distilled water. 1U of T4 DNA ligase (Promega) was then added, and the solution incubated overnight at 14°C.

2.3.4 Restriction enzyme digests

DNA was digested in the appropriate BRL REact buffer for the enzyme, using at least 2U enzyme per µg DNA (BRL). Incubations were carried out at 37°C, for either 1hr (plasmid DNA) or 3hr (genomic DNA).

2.3.5 Labelling nucleic acids

Nick Translation and random priming

Labelled plasmid DNA was prepared by nick translation (Sambrook et al., 1989). Briefly, to ~0.5µg of plasmid DNA was added, 2.5µl of 10 X Nick Translation Buffer (0.5M Tris-HCl, pH 7.5, 0.1M $MgSO_4$, 1mM DTT, 500µg bovine serum albumin; fraction V; Sigma), 20nmoles each of dATP, dGTP and dTTP (Pharmacia), 50µCi; 3000 Ci/mmole of [a-^{32}P]dCTP and the mixture was made up to 21.5µl with distilled water. After chilling (0°C) the mixture, 2.5µl of DNase I (10ng/ml; in ice-cold 1 X Nick translation Buffer containing 50% glycerol) and 2.5U of E.coli DNA polymerase I were added. The reaction was then incubated for 60 min at 16°C and stopped by the addition of 0.04 vol of 0.5M EDTA, pH 8.0. Labelled gel-purified fragments or linearised plasmids were prepared by random priming (Sambrook et al., 1989). Briefly, to ~50ng of denatured DNA (in 12µl distilled water) was added, 6µl of 4 X random priming buffer (250mM Tris-HCl, pH 8.0, 25mM $MgCl_2$, 100µM dNTPs, 1M Hepes,

pH 6.6, 27; A_{260} units/ml random hexanucleotides), 50µCi; 3000 Ci/mmole of [a-^{32}P]dCTP and 5U ofKlenow enzyme (Promega). The mixture was then incubated for at least 12 hr at room temperature and the reaction stopped by heating at 100°C for 2 min. Probes were purified by Sephadex G50 (Pharmacia) chromatography, in columns prepared from disposable 1ml syringes (Sambrook et al., 1989). Incorporation of radioactive precursor was calculated by precipitation of the acid insoluble fraction in trichloroacetic acid (TCA). Generally nick translated probes had a specific activity of 10^7-10^8 cpm/µg; random primed probes a specific activity of 10^8-10^9 cpm/µg.

DIG labelled single-stranded probes

Single-stranded antisense and sense (control) DNA probes labelled with digoxigenin were generated by thermal cycling (Patel and Goodman, 1992). The template plasmid (Kalderon and Rubin, 1988)containing the largest Type I RI cDNA in pBluescript was linearised with *Eco*RI or *Xba*I digestion prior to the reaction. The reaction contained 250ng of linearised plasmid in 0.5mM KCl, 10mM Tris-HCl; pH 8.3, 1.5mM $MgCl_2$, 0.01% (w/v) Triton X-100®, 0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.13mM dTTP, 0.07mM Digoxigenin-11-dUTP (Boehringer Mannheim), 150ng of either T7 or T3 primer, 1.25 U of *Taq* polymerase (Promega) and was incubated for 30 cycles in a Hybaid Thermal reactor (Hybaid) at 95°C for 45 sec, 55°C for 30 sec and 72°C for 1 min. The reaction product was precipitated by adding 10µg of glycogen, 0.1 vol of 3M NaOAc, pH 5.0, 3 vol of absolute ethanol and storage at -20°C for 1-2 hr. The DNA was pelleted (12,000g, 5 min) at 4°C, washed with 70% EtOH, dried and resuspended in 300µl of prehybridisation solution (5x SSPE (0.9M NaCl, 0.05M NaH_2PO_4, 5mM EDTA, pH 7.8), 50% (v/v) formamide, 5% (w/v) dextran sulphate, 1x Denhardt's solution (0.02% (w/v) Ficoll 400, 0.02% (w/v) BSA Fraction V and 0.02% (w/v) Polyvinylpyrolidone in distilled water), 250µg/ml of yeast tRNA, 500µg/ml of alkali-hydrolysed and phenol-extracted salmon sperm DNA) and used for *in situ* hybridisation without further dilution. Sense probe was generated in an identical fashion using the

appropriate primer that hybridised to the antisense strand. To evaluate the labelling reaction, to 1µl of probe was added 5µl of 5XSSC. The mixture was boiled for 5 min, cooled on ice and centrifuged for 5 min at 12,000g in a bench-top centrifuge. 1-2µl samples of the mixture were spotted onto a small nitrocellulose strip (that fitted into a 1.5ml eppendorf tube). The filter was then baked between two sheets of Whatman 3MM paper at 80°C for 30 min. After baking the filter was soaked in 2XSSPE and then washed twice for 5 min in PBT (1X PBS, 0.2% (w/v) BSA Fraction V, 0.1% (v/v) Triton X-100®). The filter strip was placed in a 1.5ml microcentrifuge tube and blocked for 30 min in PBT. The filter was then incubated for 30-60 min with the anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted at 1:2000 in PBT for 30-60 min. The filter was then washed 4 times for 15 min in PBT and finally washed twice for 15 min in 100mM NaCl, 50mM $MgCl_2$, 100mM Tris; pH 9.5; 0.1% (v/v) Tween® 20. The filter was developed with NTB and BCIP, X-phosphate as described by the manufacturers (Boehringer Mannheim). Spots were visible within a few minutes and dark by 10-15 min.

Dideoxy sequencing

Sequencing of double-stranded DNA was carried out by the dideoxy chain-termination method first described by Sanger et al., (1977). Sequencing reactions were carried out using the conditions recommended in the Sequenase Version 2.0 manual supplied by the manufacturers (United States Biochemical Corporation). For direct sequencing of PCR products the procedure described by Winship (1989) was used, primer concentration and annealing conditions were adjusted to favour primer/template annealing over template/template annealing. Reaction products were separated on 6% polyacrylamide gels, as described in section 2.3.6.

2.3.6 Size fractionation of nucleic acids

Agarose gel electrophoresis

DNA was electrophoresed on agarose gels. Generally 12cm X 13cm gels containing 100ml of agarose, which were made and run in 1 X TBE (89mM Tris-borate, pH 8.3, 2mM EDTA). A range of agarose concentrations (0.8-1.5% (w/v)) was used depending on the sizes of fragments to be resolved (Sambrook et al., 1989). Applied voltages varied between 2 and 10V/cm, depending on the time of running. Markers were usually the 123bp or 1kb ladder supplied by the manufacturers (BRL). DNA fragments were purified (Sambrook et al., 1989), from 1% (w/v) LMP (Low Melting Point agarose, BRL) agarose gels in 1 X TAE (40mM Tris-acetate, pH 7.6, 1mM EDTA) or using the Magic™ DNA purification system from Promega, using the conditions recommended by the manufacturers.

Denaturing agarose gel electrophoresis

Prior to electrophoresis, RNA samples (up to 4.5µl vol) were denatured by the addition of 10µl of formamide, 2µl of 5 X MOPS buffer (200mM MOPS, pH 7.0, 50mM sodium acetate, 5mM EDTA, 11M formaldehyde), 3.5µl of formaldehyde (12.3M), 1µl of EtBr (1mg/ml stock), and heated to 65^0C for 15 min. Prior to loading 2µl of 30% (w/v) Ficoll 400, 1mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol was added. The RNA was electrophoresed on 1% -1.2% (w/v) agarose formaldehyde gels (Sambrook et al., 1989), using 1 X MOPS, with constant circulation from anode to cathode chambers in order to maintain a constant pH.

Denaturing polyacrylamide gel electrophoresis for sequencing

Products of DNA sequencing reactions were separated on denaturing polyacrylamide gels: 6% (w/v)acrylamide (Acrylamide: N, N'-methylenebisacrylamide, 19: 1), 7M urea, in 1 X TBE. Polymerisation was initiated by the addition of 1ml of 10% (w/v) ammonium persulfate and 20µl of TEMED (N, N, N' N', -tetramethylenediamine) to 150ml of 6% acrylamide/urea. Gels were allowed to polymerase overnight before use and were pre-run at 100W for 45-60 min in 1 X TBE. Samples were denatured for 2 min at 75°C in 1 X sequencing gel loading buffer (as supplied in Sequenase Version 2.0 Kit), and then loaded onto the gel. Gels were run for various lengths of time depending on the sizes of DNA to be resolved, and then dried for 1-2 hr at 80°C onto Whatman 3MM paper under vacuum. Autoradiography was carried out without intensifying screens at room temperature (see section 2.3.9).

2.3.7 Visualisation and photography of gels

DNA and RNA were visualised by UV induced fluorescence on either a short wave (254nm) or long wave (302nm) transilluminator after staining agarose gels with EtBr or adding EtBr to the sample (0. 5µg/ml). Gels were photographed using either a Polaroid camera loaded with 545- or 667- land film and fitted with a Kodak Wratten filter No. 23A, or with a Pentax 35mm SLR camera fitted with a Kodak Wratten filter No. 23A, using Ilford HP5 film.

2.3.8 Blotting and nucleic acid hybridisation

Southern blotting and DNA/DNA hybridisations

Agarose gels containing DNA were transferred to nylon membranes (Hybond-N), either by capillary action or vacuum blotting (Stratagene, Posiblotter©), and fixed to the

membrane by UV treatment as instructed by the manufacturer (Amersham UK). DNA/DNA hybridisations were carried out at 65°C in 5 X SSPE, 10X Denhardt's solution, 1% (v/v) SDS, 0.005% (w/v) sodium pyrophosphate and 100µg/ml of denatured sonicated salmon sperm DNA. Filters were pre-hybridised at 65°C for at least 1 hr before addition of the denatured radioactive probe (10^5-10^6 cpm/ml of hybridisation solution) and hybridised for a minimum of 16 hr. After hybridisation, the filters were washed in 2 X SSC at room temperature for 15 min, 1 X SSC, 0.1% SDS at 65°C for 15 min and then a final wash of 0.1 X SSC, 0.1% SDS at 65°C for 15 min. Prior to autoradiography, filters were blotted dry and covered in Saran Wrap™.

Northern blotting and DNA/RNA hybridisations

Agarose formaldehyde gels containing RNA were transferred to Reinforced nitrocellulose (Hybond C+), by capillary action (as recommended by Amersham). RNA was fixed to the membrane by baking for 2 hr at 80°C. DNA/RNA hybridisations were carried out at 42°C in 50% (v/v) formamide, 5 X SSPE, 2 X Denhardt's solution and 0.1% SDS. Filters were pre-hybridised at 42°C for at least 3 hr before addition of the denatured radioactive probe (10^5-10^6 cpm/ml of hybridisation solution) and hybridised for a minimum of 16 hr. After hybridisation, the filters were washed in 1 X SSPE, 0.1% SDS at room temperature for 20 min and then 3 times in 0.2 X SSPE, 0.1% SDS at 65°C for 20 min. Prior to autoradiography, filters were blotted dry and covered in Saran Wrap™.

2.3.9 Autoradiography

Autoradiography of probed filters was carried out at -70°C, where necessary with intensifying screens and exposed to Fuji NIF RX X-ray film, for as long as required. Films were developed using a Kodak X-Omat film processor.

Chapter 3

"Site-selected" transposon mutagenesis of *Drosophila.*

3.1 Introduction

Classical *Drosophila* genetics is based on the generation of mutants with interesting phenotypes followed by isolation and characterisation of the gene(s) involved i.e. mutation—phenotype—gene (Rubin, 1988; Ashburner, 1989a). In *Drosophila* mutations can be induced by a large number of mutagenic agents. Notable examples being chemical mutagens such as ethyl methane sulfonate (EMS) which induces mutations that are almost exclusively point mutations, irradiation which induces chromosomal aberrations (translocations, inversions, deletions) or mobilisation of transposable elements (eg. P-elements) to produce mutations in regulatory or controlling regions of genes (Grigliatti, 1986; Kidwell, 1986). It is then possible to screen systematically for all genes that can mutate to produce a given phenotype. Screens for mutations affecting a wide range of processes can be devised, based on any phenotypic criteria. A gene identified by its mutant phenotype can then be cloned by a variety of efficient molecular strategies (Pirrotta, 1986; Rubin, 1988; Ashburner, 1989a). These cloning strategies exploit the fact that genes in *Drosophila* can be mapped to a precise physical location, by cytological localisation to polytene chromosomes (Pardue, 1986). The rich array of *Drosophila* stocks with various chromosomal rearrangements enables the localisation of a mutant gene to within a few chromosome bands (Lindsley and Zimm, 1992).

With advances in recombinant DNA technology, we are increasingly faced with the problem of assigning a function to an ever growing number of genes that have been cloned and characterised, without any reference to phenotype (Levy et al., 1982; Palazzolo et al., 1989). The first hint about gene function can come from sequence comparisons, a second from a detailed study of their patterns of expression. This can offer an abundance of circumstantial evidence about function, but in general, without an alteration of the gene and analysis of mutant phenotypes, we are severely impeded. Thus there is a need to develop an approach that will generate mutants in selected genes to

determine their function. This is the basis of "reverse genetics", i.e. gene—mutant—phenotype.

The standard approach is to inactivate the gene and see what effect this has. The function of a gene can, in principle, be inactivated at three levels: Protein, RNA, and DNA . All of these approaches have been applied to *Drosophila* with varying degrees of success. This may include the production of "dominant negative" mutations (Herskowitz, 1987) in which the function of a gene is blocked at the protein level: the cloned gene is altered so that it encodes a mutant product capable of inhibiting the wild-type gene product in a cell (Drain et al., 1991; Griffith, 1993). The use of "antisense" RNA, to block the expression of a gene by preventing the translation of its sense transcripts, has been successfully demonstrated (Rosenberg et al., 1985). A more recent approach has been the use of "designer" ribozymes (RNA enzymes) where specific ribozymes can be designed for cleavage of specific RNAs, allowing a new approach to gene inactivation (Heinrich, et al., 1993). In yeast and mammalian cells a wild-type gene can be replaced by a mutant version by homologous recombination (Botstein and Fink, 1988; Kim and Smithies, 1988), but in *Drosophila*, gene replacement by homologous recombination has not been demonstrated. As such there is a need to develop a reverse genetics approach that will generate mutants in any gene of interest.

This chapter describes the development of a "reverse genetics" approach to this problem which exploits the sensitivity of the polymerase chain reaction (PCR; Saiki et al., 1988) to isolate single appropriate transposon insertion mutants within a large population of mutagenised flies. The transposable element of choice in *Drosophila* is the P-element (see section 1.8). The advantages of P-elements as mutagenic agents are largely based on the frequency and ease with which they can be mobilised in a controlled manner in a dysgenic cross. The P-M system has the potential to induce insertions, deletions, excisions and chromosomal rearrangements. Suitable dysgenic crosses can be set up and the new mutations can be stabilised or, if appropriate, P-element insertions in or near a gene of interest can be remobilised to recover new alleles of the gene through imprecise excision and chromosome rearrangements(see section 1.8.4).

Presented in this chapter is the theory and demonstration of a reverse genetics approach, termed "site-selected" transposon mutagenesis; which allows the detection and isolation of rare individuals within a population of mutagenised flies that carry a transposable P-element inserted into a target gene.

3.2 Theory behind "Site-selected" transposon mutagenesis.

P-elements are a family of transposable elements found in *Drosophila melanogaster*, the mobilisation of which can be manipulated experimentally by an appropriate genetic cross (Engels, 1989). Frequencies of P-element mutagenesis are somewhat locus-dependent, varying from as much as 10^{-2} to as little as 10^{-7} (Kidwell, 1987; Engels, 1989). Nonetheless, by using engineered P-elements, Robertson et al., 1988, observed ~10 new insertions per mutagenised genome. Since the estimated *Drosophila* genome size is ~1.65 X 10^5 kb, the average probability of insertion every 1 kb along the genome would be on the order of 10^{-4} per fly. If the mutagenised population is sufficiently large (10^4 flies), there is a significant probability that any gene could be a target for a P-element insertion.

The sensitivity of the polymerase chain reaction is such that (Saiki et al., 1988) it is possible to detect the occurrence of a single appropriate P-element transposon insertion among a population of mutagenised flies, as shown in Figure 3.1. Here, the juxtaposition of a P-element and the specific gene of interest provides a unique substrate for amplification by PCR. P-elements are bounded by 31 bp inverted repeats, so regardless of the orientation of a P-element at a given locus, a PCR primer derived from this 31 bp sequence, in conjunction with a gene-specific primer (GSP) can be used to amplify gene-specific DNA. Amplification will therefore lead to an exponential increase of the product which has its ends defined by the positions of the GSP and P-element primers. *Bona fide* amplification products can be distinguished from non-specific amplification products by their ability to hybridise with a cloned fragment of the target gene. In addition, sequencing of the PCR product should demonstrate juxtaposition of target and P-element sequences.

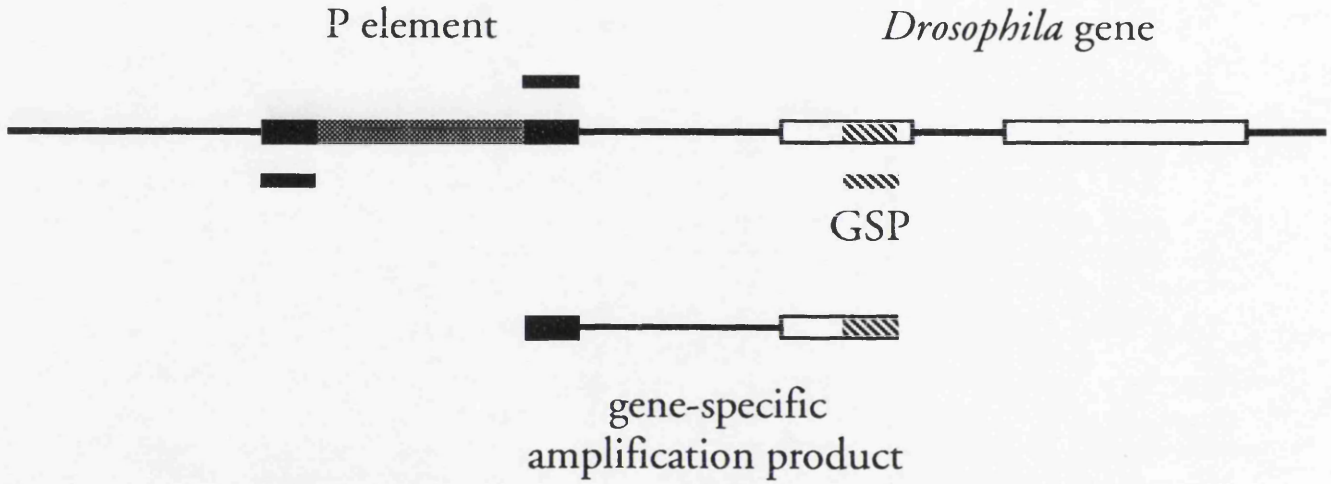


Figure 3.1. Detection of a P-element transposon insertion into a gene of interest. Exponential amplification occurs only when a P-element inserts near the gene-specific primer (GSP), creating a unique substrate for amplification by PCR (whose ends are defined by the positions of the GSP and P-element primers). Open boxes represent exons of the hypothetical *Drosophila* gene. (Diagram taken from Sentry et al., 1993a).

Mutagenised flies are screened by carrying out the PCR on DNA extracted from their eggs. The rare fly can be identified by using a "sib-selection" strategy (see section 3.4.2) of repeatedly subdividing populations and testing for a fly with the desired P-element insertion.

This technique requires no *a priori* hypothesis concerning the expected phenotype as individuals are isolated on the basis of their structural properties, not their mutant phenotype. In addition the selected individuals are isolated as heterozygotes, allowing isolation of recessive lethal phenotypes.

3.3 Detection of known P-element insertions at the *sn* locus: CFL3, CFL5

To examine the feasibility of this technique to detect insertions in the *Drosophila* genome, experiments were first done with mutations known to have been induced by the insertion of a P-element into the *singed* gene which is located at cytological position 7D1-2 on the X chromosome. The *singed* gene has been cloned, characterised, sequenced and mutant alleles of the gene result in defects in bristle morphology and female fertility of varying severity (Figure 3.2a & 3.2b). *sn* is a known "hot-spot" for P-element insertion and in some mutants the exact position and orientation of the P-element insertions are known (Roiha et al., 1988). Figure 3.3 illustrates the 5' regulatory region of the *sn* gene. The "hot-spot" region is less than 700 bp and possibly only 100 bp and several independent sites of insertion have been documented in this region (Roiha et al., 1988). Strains CFL3 and CFL5 have P-elements at different sites and orientations within the hot-spot of Figure 3.3 (Roiha et al., 1988).

The nucleotide sequence of the *sn* gene (Jamie Paterson and Kevin O'Hare, pers. comm.) was used to design an appropriate oligonucleotide primer (snA) that would theoretically detect a P-element insertion in the "hot-spot" region. The nucleotide sequence of the P-element (O'Hare and Rubin, 1983) was used to design an oligonucleotide primer to the terminal inverted repeat of the P-element (Figure 3.4; P_{31}). Figure 3.5a shows the amplification products and hybridisation results of mutant CFL3

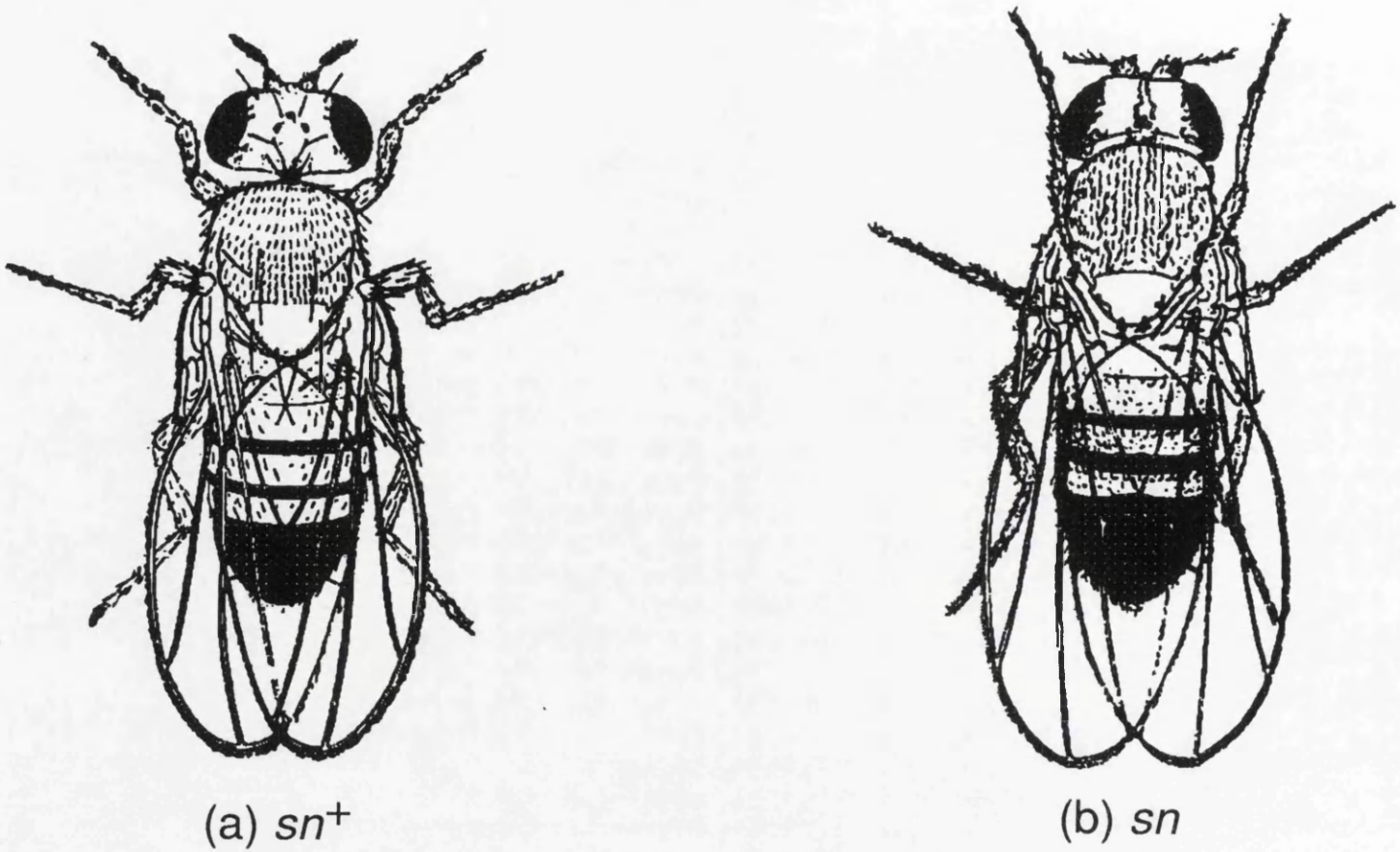


Figure 3.2. Illustrations of bristle phenotypes (a) wild-type (b) *sn.* (Taken from Lindsley and Zimm, 1992).

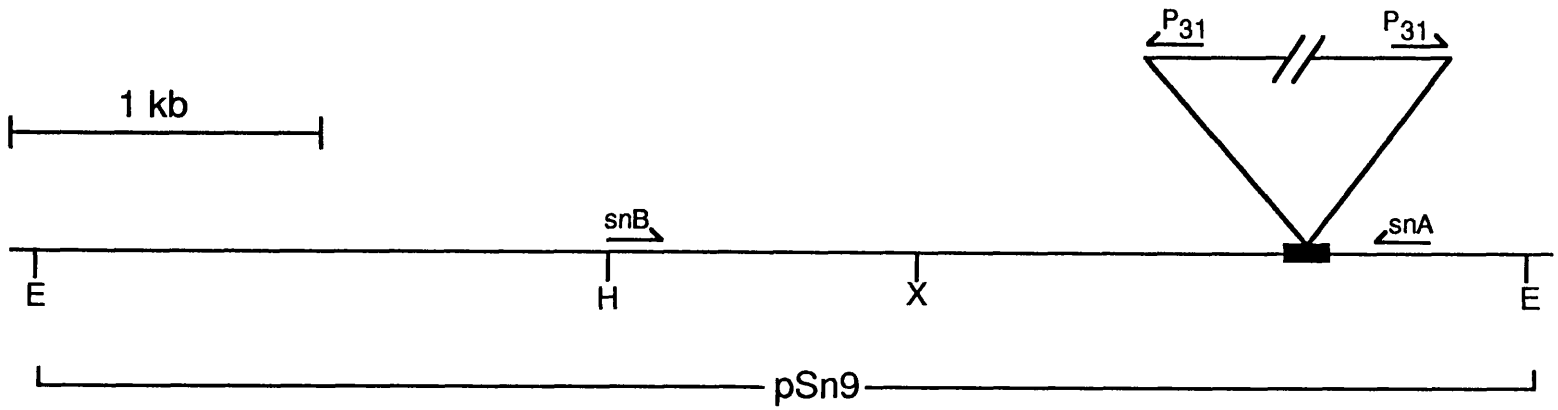


Figure 3.3. 5' regulatory region of the *sn* gene. The shaded area represents the 100 bp "hot-spot" region, where several (at least 10; Roiha et al., 1988) independent sites of P-element insertion have been observed. (E) *Eco*RI; (H) *Hind*III; (X) *Xba*I. PCR primers are shown as arrows (5'→3' orientation). Triangle represents P-element insertion. pSn9 is a cloned *Eco*RI fragment. (Diagram adapted from Kaiser and Goodwin, 1990).

Choice of P-element specific Primers

Left end:

```
          10          20          30          40          50          60
CATGATGAAA TAACATAAGG TGGTCCCGTC GAAAGCCGAA GCTTACCGAA GTATACACTT
GTACTACTTT ATTGTATTCC ACCAGGGCAG CTTTCGGCTT CGAATGGCTT CATATGTGAA
```

Right end:

```
          2860        2870        2880        2890        2900
AA GCATACGTTA AGTGGATGTC TCTTGCCGAC GGGACCACCT TATGTTATTT CATCATG
TT CGTATGCAAT TCACCTACAG AGAACGGCTG CCCTGGTGGA ATACAATAAA GTAGTAC
```

5' CGACGGGACCACCTTATGTTATTTCATCATG 3' P_{31}

5' GTGTATACTTCGGTAAGCTTCGG 3' P_{Left}; P_L

5' AGCATACGTTAAGTGGATGTCTC 3' P_{Right}; P_R

Figure 3.4. DNA sequence of the left and right ends of the 2.9 kb P-element. Primers chosen are indicated by an appropriate colour. All internally deleted elements retain, at least, *cis*-acting sequences up to position 162 and beyond position 2686. (Sequence taken from O'Hare and Rubin, 1983; in the EMBL database (Heidelberg) under accession No. V01520.

and CFL5 DNAs amplified with P_{31} and snA primers and hybridised with the pSn9 clone. The predicted sizes of the amplification products with P_{31} and snA primers are 400 bp and 378 bp, respectively. Amplification of CFL3 DNA with P_{31} and snA occasionally produces an artefactual band (Figure 3.5a) that also hybridises to the pSn9 probe. In the experiments described in this chapter, the P_{31} primer was used in conjunction with the appropriate gene specific primer.

In experiments carried out after this mutagenesis was concluded, described in Chapter 4, two new P-element primers, P_{Left} and P_{Right} (P_L and P_R; Figure 3.4) were utilised. These primers give the orientation of the P-element insertion with respect to the gene-specific primer. Figure 3.5b shows the amplification products and hybridisation results of mutant CFL3 and wild-type Oregon R DNAs amplified with snA and either P_L or P_R primers individually or in combination. Amplification was dependent upon the gene-specific primer (snA) and only one P-element primer, P_L (the orientation and size of the P-element insertion at *sn* in CFL3 has been previously determined to be a 2.9 kb P-element insertion, transcribed in the opposite orientation to the *sn* gene, Roiha et al., 1988). Although using three rather than two primers increases the complexity of the PCR reaction, it was fortuitous that P_L and P_R used in combination produce less artefactual amplification than P_{31}/snA combinations. These artefactual amplification products are produced in all strains tested, even when the primers are used individually, but not in their absence or in the absence of DNA (data not shown).

P_{31} and snA primers, either in combination or individually were used to amplify DNA isolated from several strains: CFL3 (*sn* mutant), from the sn^+ and M strains Oregon R, Canton S and m56i, and from strains that carry nonautonomous and autonomous P-elements, *Birmingham-2* (17 P-elements) and Harwich (~40 elements). Amplification products were visualised on a ethidium bromide-stained gel, but the *bona fide* product of CFL3 DNA was detected only by hybridisation with the pSn9 probe (Figure 3.6). Thus, under these conditions, the generation of artefactual amplification products appears to be independent of the number of P-elements in the genome.

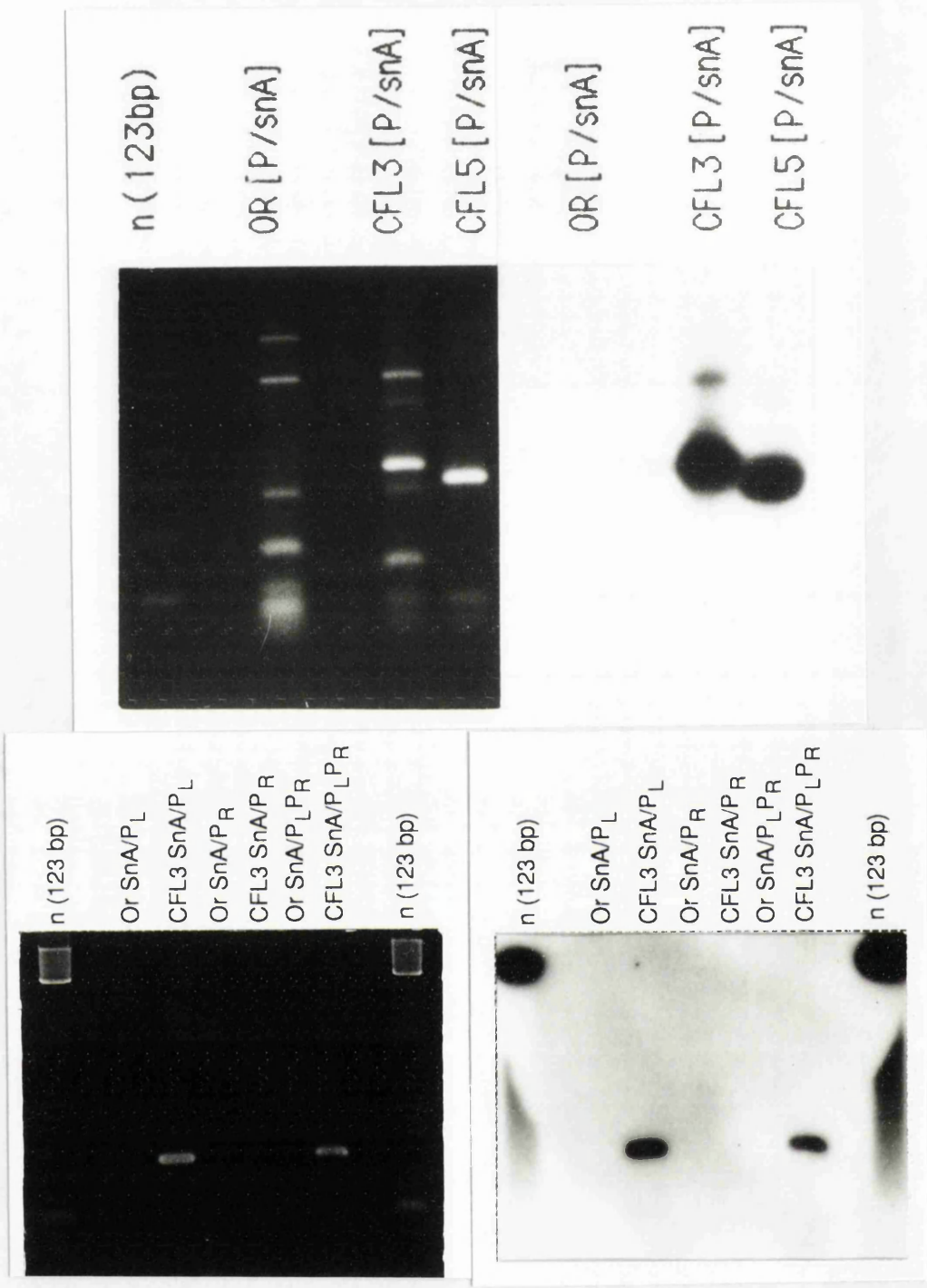


Figure 3.5. P-element-dependent amplification of the *sn* region.

(a) Amplification was with P_{31} and snA primers and probed with pSn9. *sn* mutants CFL3 and CFL5 have P-elements at different sites within the "hot-spot" of Fig. 3.3. n (123bp) corresponds to 123bp DNA size marker (BRL). (*Left*) Ethidium bromide stained gel, 1.5% agarose. (*Right*) Autoradiograph.

(b) CFL3 or Oregon R (OR) DNA was amplified with snA and either P_L or P_R primers individually or in combination. (*Left*) Ethidium bromide stained gel, 1.5% agarose. (*Right*) Autoradiograph.

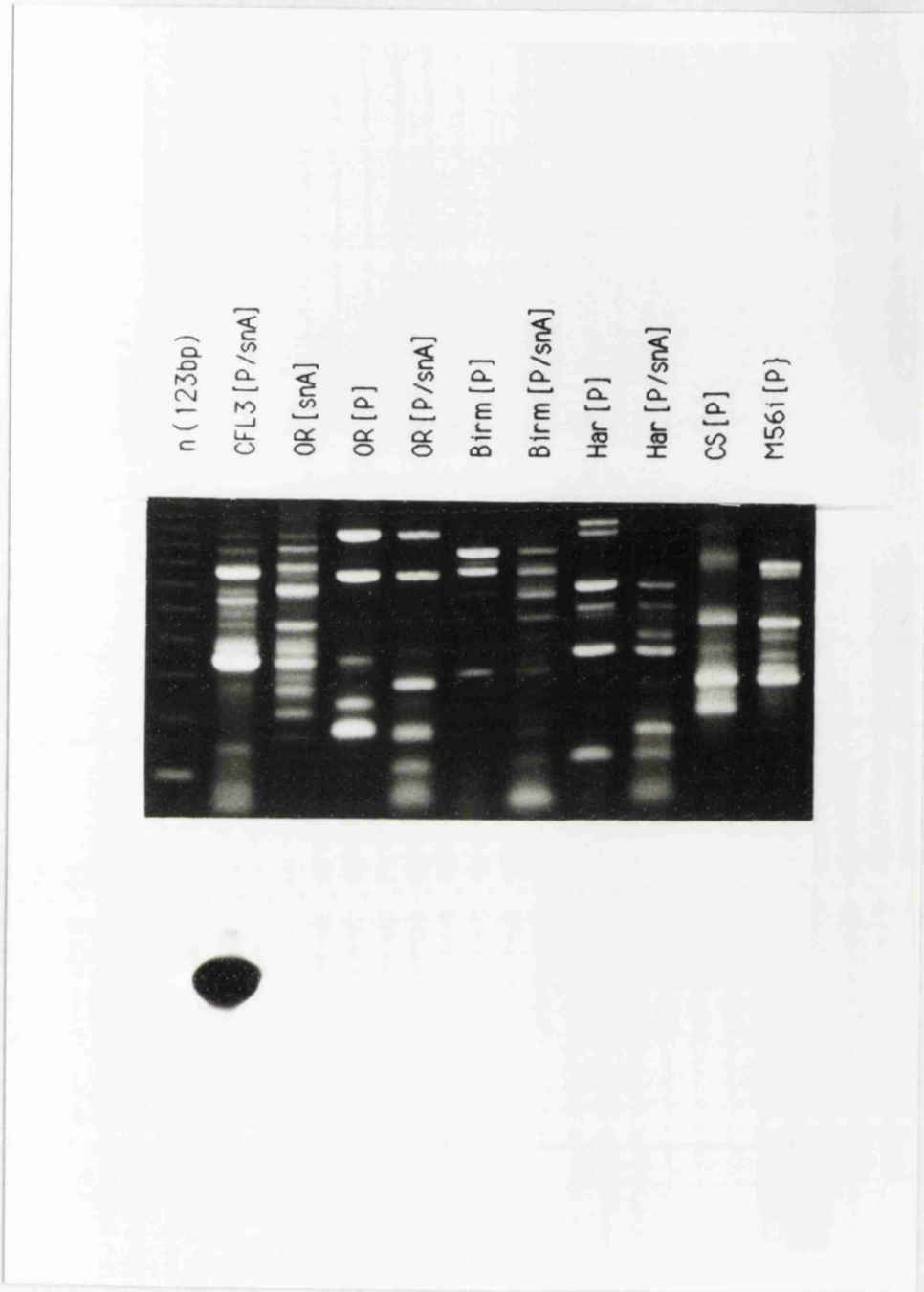


Figure 3.6. Specific and artefactual amplification products from different *Drosophila melanogaster* strains. P_{31} and snA primers, either alone or in combination, were used to amplify DNA isolated from the *sn* mutant CFL3, from the sn^+ and M strains Oregon R (OR), Canton S (CS), and M56i, and from P strains, *Birm-2* (Birm) and Harwich (Har). Amplification products were observed in all cases (*Upper*), only the 400 bp *bona fide* product of CFL3 DNA was detected by hybridisation with the pSn9 probe (*Lower*). (*Upper*) Ethidium bromide-stained gel, 1.5% Agarose gel. (*Lower*) Autoradiograph.

Production of artefactual products would also appear to be independent of the PCR annealing temperature (Figure 3.7). Dr. Kim Kaiser (pers. comm.), in an attempt to improve the reaction specificity, amplified DNA from Oregon R and CFL5 mixed 1:1, with P_{31} and snA primers, at three different annealing temperatures (50°C, 60°C and 70°C) (Figure 3.7). We are unsure of the source of these artefactual products, but some might arise, stoichastically, by "self-priming" from sequences within the genome that are partially homologous to the primers and their production varies from experiment to experiment. Fortunately these products share no homology to the *sn* locus and so do not confuse the analysis. During a mutagenesis (this chapter and chapter 4), one factor seemed to be the amount of heterogeneity in the sample; when pools of individuals became smaller the amount of background also decreased.

A reconstruction experiment was carried out to test if it was possible to detect a sn individual in DNA isolated from a population of flies in which only one individual per thousand was of the desired type. A serial dilution (1:1, 1:10, 1:100 and 1:1000) of CFL5 DNA into wild-type Oregon R DNA was amplified with P_{31} and snA and gave the predicted 400 bp amplification product after 30 cycles. The 400 bp product can be visualised on a ethidium bromide-stained gel at 1 fly in 100. Furthermore, under these conditions, 1 fly in 1000 can be detected by hybridisation with a gene-specific probe (pSn9; Figure 3.8).

3.4 "Site-selected" transposon mutagenesis of the *singed* locus

Given that new mutations occur at *sn* during a hybrid dysgenic cross at frequencies of up to 1% (Roiha et al., 1988), the technique of "site-selected" mutagenesis was tested by isolating new P-element induced mutations of *sn*. As discussed in the previous section, the *sn* gene has a known "hot-spot" region and the gene-specific primer, snA, was used to detect insertions in this region (Figure 3.3).

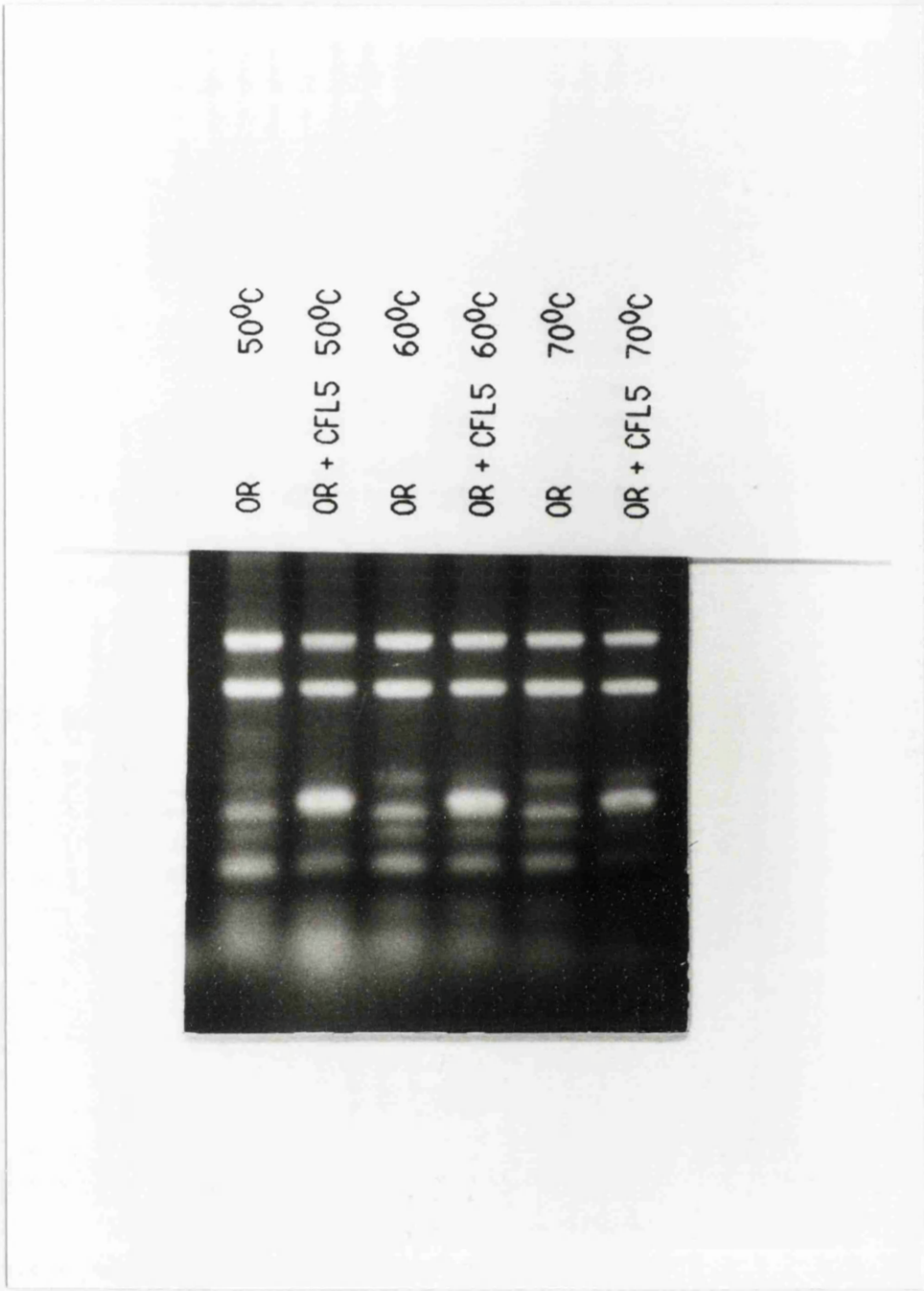


Figure 3.7. Oregon R and Oregon R DNA "seeded" 1:1 with CFL5 DNA amplified with P_{31} and snA primers at three different annealing temperatures (50°C, 60°C and 70°C). Amplification products were separated on a 1.2% agarose gel stained with ethidium bromide. (Experiment by Dr Kim Kaiser, with permission).

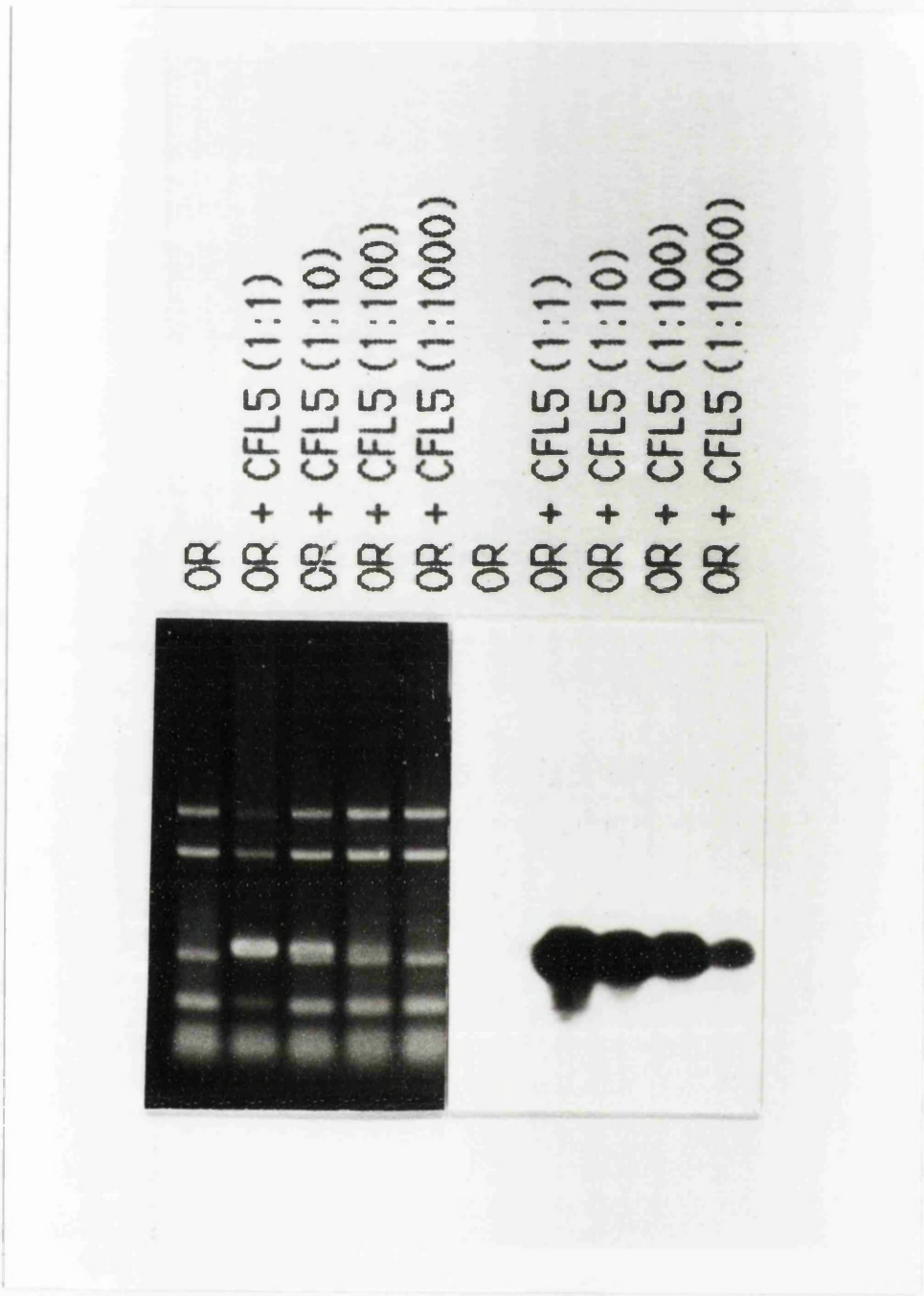


Figure 3.8. A serial dilution (1:1, 1:10, 1:100, 1:1000) of CFL5 egg DNA into Oregon R (OR) DNA and amplified with P_{31} and snA primers. After 30 cycles *Bona fide* amplification products are easily detected by hybridisation with pSn9, down to a dilution of 1:1000. (*Left*) Ethidium bromide-stained gel, 1.5 % agarose. (*Right*) Autoradiograph.

3.4.1 P-element mutagenesis

To generate random P-element transpositions, a hybrid dysgenic cross was carried out as described in Robertson et al., 1988. Briefly, *Birm-2; ry* ^{506} males (homozygous for a second chromosome bearing 17 defective P-elements) were mated at 16°C with *w;Sb P*[*ry* $^{+}$Δ2-3](99B)/TM6 females. *P* [*ry* $^{+}$Δ2-3] provides a nonmobilisable source of transposase, from which intron 3 of the transposase gene has been removed *in vitro* (Laski et al., 1986). Δ2-3 expresses transposase at high levels. F1 *Sb* male progeny were crossed to wild-type females at 18°C. This reduces the fertility problems associated with flies bearing *Birm-2* and Δ2-3. There are two parameters that affect fertility: age and temperature. As the flies used in this cross age, their fertility drops off quickly. After three days (at 21°C) it is about 50% and after a week they are almost completely sterile (Phillis, pers. comm.). Thus for the best fertility F1 *Sb* male progeny were used as soon as possible after eclosion and never exposed to temperatures above 19°C. The resulting F2 *Sb*$^{+}$ mutagenised females were divided into batches of 100 with approximately 20 wild-type males. This particular crossing scheme is extremely efficient due to the presence of the Δ2-3 element and 17 defective P-elements so that the maximum possible insertion rate per F2 genome was generated, producing on average approximately 10 new insertions per mutagenised genome (Figure 3.9; see section 1.8.3). Despite the ability of the Δ2,3 element to stimulate high levels of transposition, it is itself nonmobilisable so that chromosomes with new insertions that segregate away from the Δ2-3 bearing chromosome should be stable.

3.4.2 Sib selection

Non-destructive testing of the mutagenised populations was carried out using a "sib selection" strategy (Figure 3.10; Kaiser, 1990). This strategy exploits the fertility of the fly and good fly husbandry in order to isolate individuals of interest. Batches of up to 1000 mutagenised females are introduced to wild type males, matured for several days

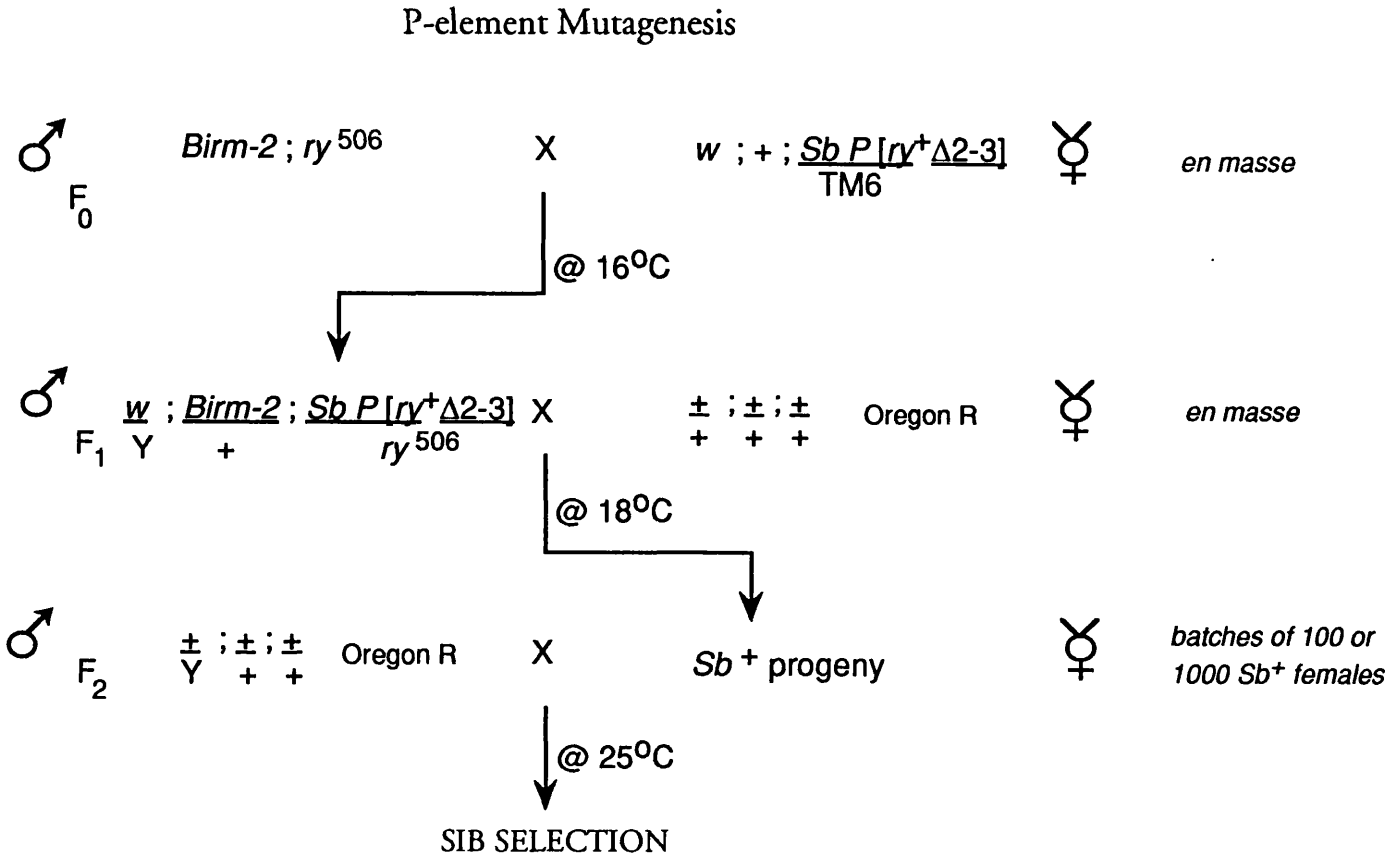


Figure 3.9. P-element mutagenesis strategy. as described by Robertson et al., 1988 (see section1.8.3 and Figure 1.9).

SIB-SELECTION

Day	1-2:	Harvest 10^3 virgin females and introduce to wild-type males										
Day	3:	Age for one day										
Day	4:	Sub-divide 10-fold Collect eggs and isolate DNA										
Day	5-6:	PCR	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2	10^2
Day	7:	Sub-divide 10-fold Collect eggs and isolate DNA			I							
Day	8-10:	PCR	10	10	10	10	10	10	10	10	10	10
Day	11:	Establish single lines						I				
Day	22:	Sacrifice females and isolate DNA										
Day	27:	PCR	1	1	1	1	1	1	1	1	1	1

Half the progeny of a selected female breed true for insertion in the target gene. Balance insertion with appropriate balancer strain.

Figure 3.10. The "sib selection" strategy, indicating how a single fly with the desired characteristics can be isolated from a population of 10^3 mutagenised females via repeated cycles of subdivision and PCR. (Figure adapted from Kaiser, 1990).

and then allowed to lay eggs for 24 hours. Thus, by carrying out PCR on the egg DNA, it is possible to recognise one or more females that are laying an egg with a P-element insertion in the desired region, and screening heterozygotes avoids any effects that the P-element insertion might have on viability of the fly. The F2 females are then sub-divided as in Figure 3.10 and PCR is carried out as before allowing the female of interest to be narrowed down to single fly. Thus by a process of elimination, it is possible to isolate the female laying the "Golden egg". This female is then allowed to lay in a vial and her progeny will carry the desired insertion. Consequently, by crossing to appropriate balancer stocks (eg : attached-X, see table 2.1, chapter 2) the insertion can be maintained for further studies.

A population of 900 F2 females produced by the dysgenic cross described in section 3.4.1 (Robertson et al., 1988) was placed in a cage and allowed 24 hours to mature before egg collection and DNA isolation. Over the next 3 days, DNA was isolated from their eggs and screened by amplification with P_{31} and snA primers. A specific amplification product was detected by southern hybridisation using the pSn9 probe (Figure 3.11, lane 1) and the 900 females were immediately divided into batches of 100. Each batch was placed in a small cage (with approximately 20 wild-type males) and egg collection was continued. By a process of elimination, the population had been reduced to three batches of 100 females; batches 1, 2, and 6 (Figure 3.11, lanes 2, 3 and 7). Yet again, each of the three batches of 100 was subdivided into groups of 10 and eggs collected the following day. When the population had been narrowed down to three batches of 10, batches 1.9, 2.7, and 6.5 (Figure 3.12), the groups of 10 were subdivided into single females, placed into individual vials with several wild-type males and allowed to generate progeny. DNA was isolated from half of the female (F3) progeny produced by each individual of a positive batch and amplified with P_{31} and snA primers. An amplification product of appropriate size which was indicative of a P-element insertion at the *sn* gene was detected in the daughters of female 1.9.3 (Figure 3.13), and in the daughters of females 2.7.2 and 6.5.10 (data not shown). In accordance with the selection criteria employed, *sn* males were found in the positive vials (1.9.3, 2.7.2 and

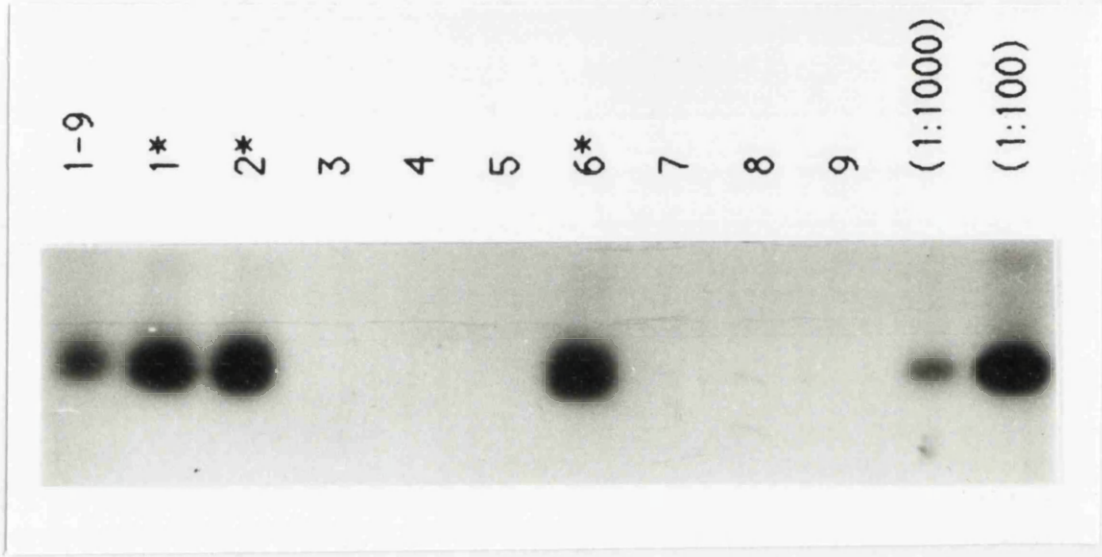


Figure 3.11. Detection of putative P-element insertions at the *sn* gene. Lanes: The lane (labeled 1-9) represents amplified egg DNA from a batch of 900 F2 dysgenic females. The next nine lanes represent amplified DNA from eggs laid by the 9 sub-divided batches of 100 F2 females. The last two lanes are dilutions of CFL3 DNA in Oregon R DNA (1:1000) and (1:10). All DNAs were amplified with P31 and snA primers, separated in a 1.5% agarose gel, blotted and hybridised with pSn9. Positive females with P-element insertions at the *sn* gene were detected in batches 1, 2, and 6.

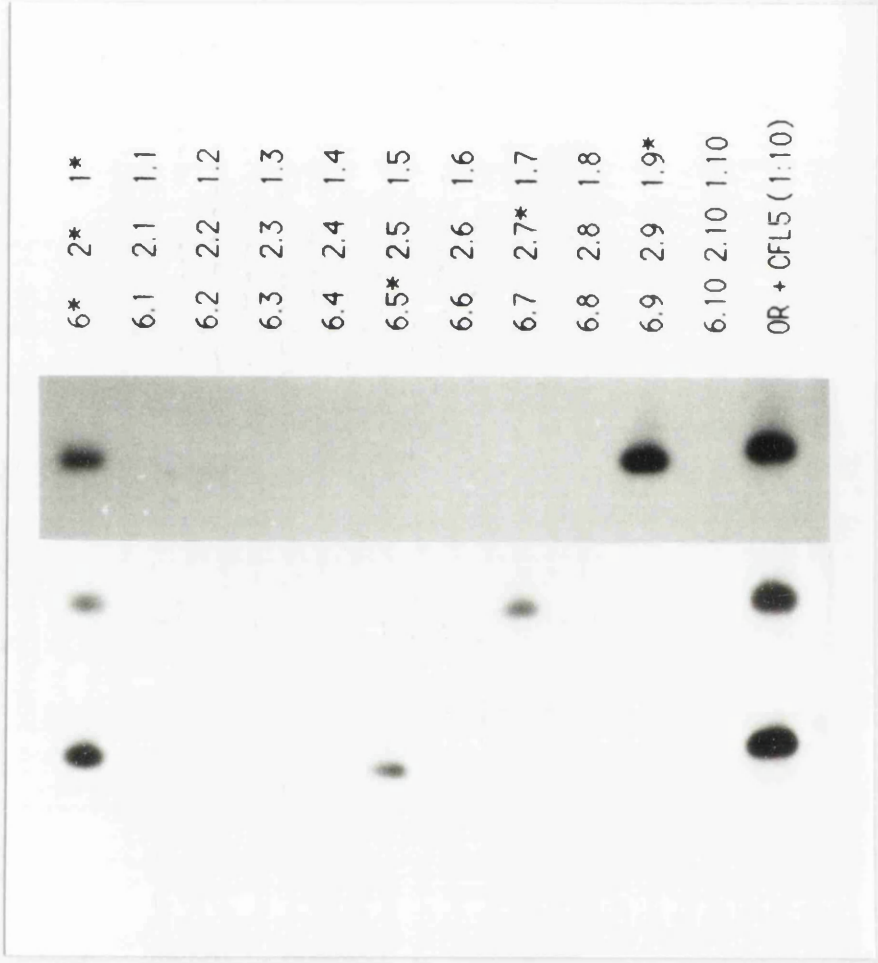


Figure 3.12. Further subdivision of batches 1, 2, and 6. Females with P-element insertions at the *sn* gene were detected in batches 1.9, 2.7. and 6.5. (*Top*) Subdivision of batch 1. (*Middle*) Subdivision of batch 2. (*Bottom*) Subdivision of batch 6. Each panel is an autoradiograph of a blot probed with pSn9.

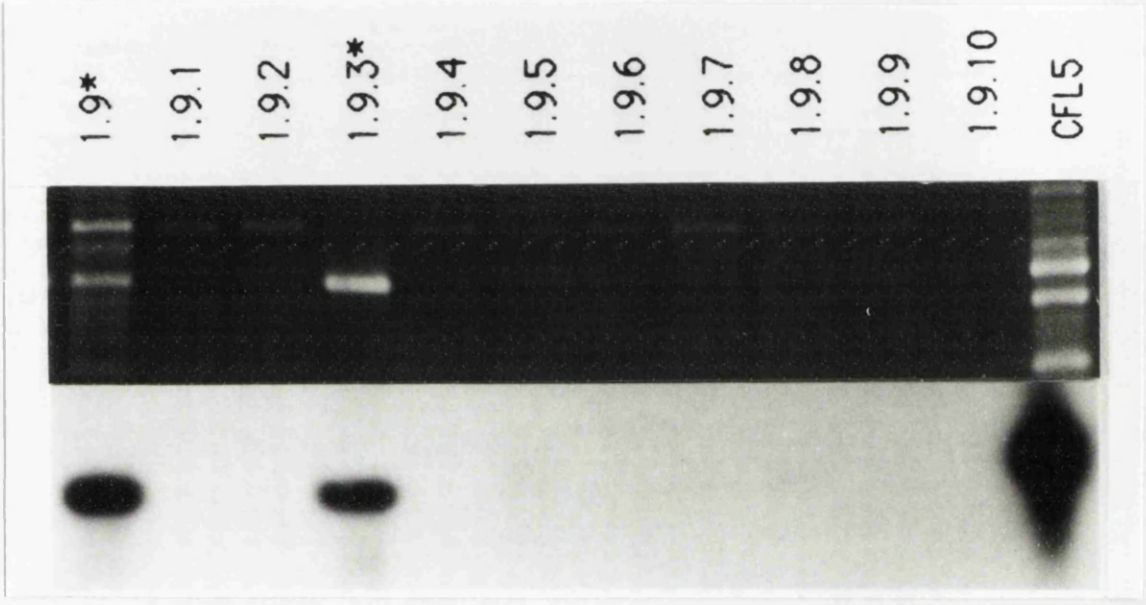


Figure 3.13. An example of further subdivision of batch 1.9. DNA was isolated from half of the female (F3) progeny produced by each individual of batch 1.9. and amplified with P_{31} and snA primers. A positive amplification product was detected in the daughters of the female 1.9.3. (*Upper*) Ethidium bromide-stained gel, 1.5% agarose. (*Lower*) Autoradiograph, probed with pSn9.

6.5.10) and comprised approximately half of the total male progeny (Figure 3.14; their *sn*$^+$ brothers were discarded without further analysis). These *sn* lines were designated snG4, snG5 and snG6. Figure 3.15 summarises the results of this mutagenesis strategy; size differences between amplification productsare indicative of different sites of insertion within the "hot spot" region of Figure 3.3. Hybridisation with the pSn9 probe reveals only the predominant amplification products shown in the figure 3.15.

DNA from wild-type Oregon R, CFL3, CFL5 and *sn* males (snG4-6) was digested with *Eco*RI and *Hin*dIII, separated in a agarose gel, blotted and probed with pSn9. Two *Eco*RI-*Hin*dIII fragments are observed in the wild-type, Oregon R DNA. Southern blot analysis (Figure 3.16) revealed that the 3 kb *Eco*RI-*Hin*dIII fragment (that spans the hot spot shown in Fig. 3.3) has been disrupted in the mutant lines and the size of the 1.9 kb fragment remains intact. As an internal control, batches of females not selected by the above criteria were tested for their ability to generate *sn* progeny. None of the unselected females produced *sn* progeny. Genetically all of the *sn* mutations (snG4-6) are allelic with CFL3. The P-element insertions associated with the new *sn* mutations segregate with the X-chromosome, since they are not inherited by the female progeny of a cross between *sn* males and attached X females.

Final verification that the *sn* mutations were indeed caused by P-element insertions came from amplification with P_L and P_R primers, allowing orientation of the P-element insertion with respect to the gene-specific primer. snG4-6 DNAs were amplified with snA and either P_L or P_R primers individually. Amplification of each PCR product was dependant upon the gene-specific primer (snA) and only one P-element primer (Figure 3.17; snG4, P_R; snG5, P_L; snG6, P_R). The DNA sequence of the junction between the P-element insertion and *sn* was determined for all three mutants. The PCR products from figure 3.17 were purified and cloned into *Eco*RV cleaved, T-tailed pBluescript®II KS- plasmid vector (see section 2.3.3) and sequenced with T3 and T7 primers and the results are shown in Figure 3.18. Sequence analysis of snG4-6 confirms that P-elements have inserted in the hot-spot region and the sequence-specific nature of P-element insertion previously observed at *sn* (Roiha et al., 1988). The

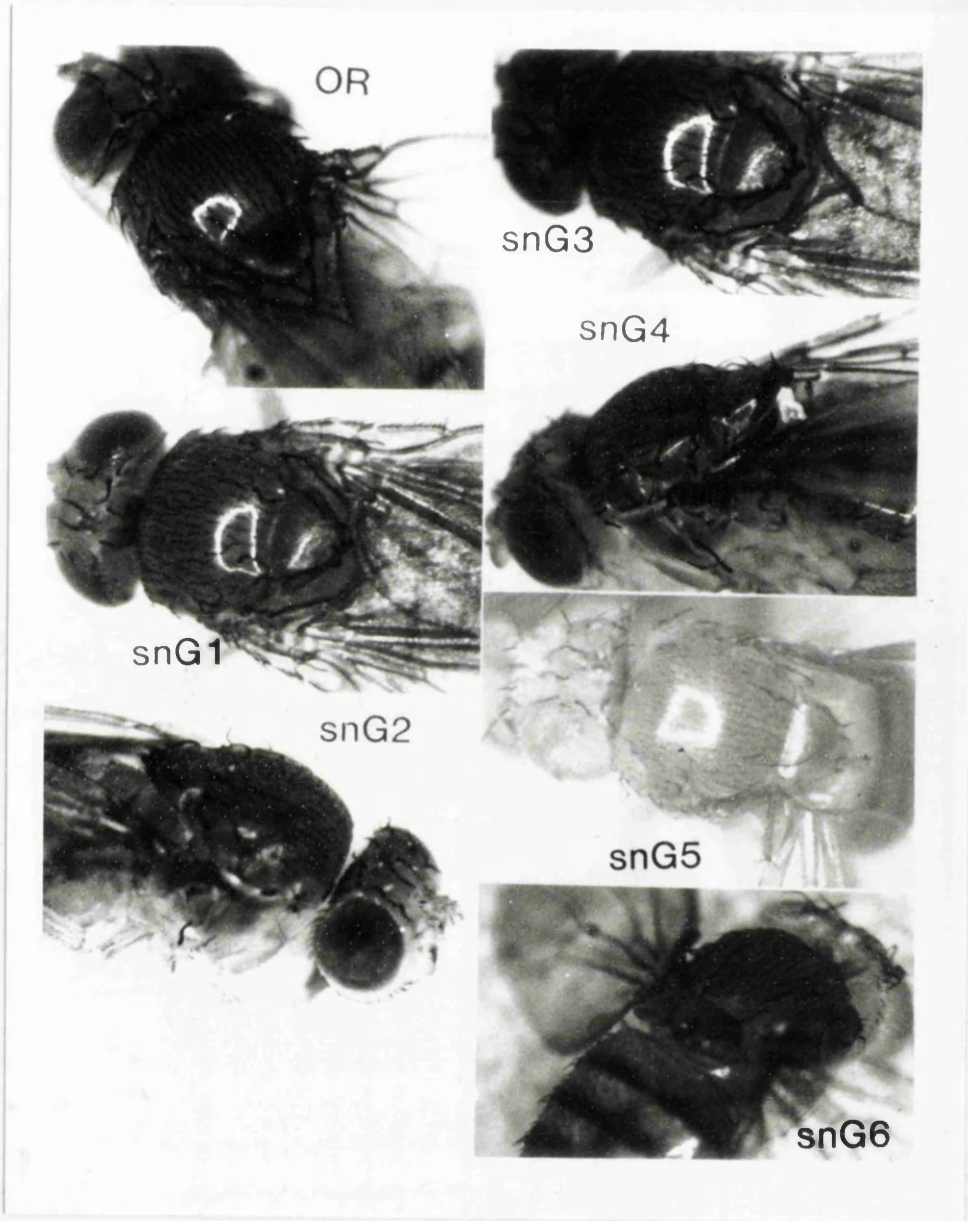


Figure 3.14. Bristle phenotypes of the male snG4-6 mutants compared with wild-type, Oregon R (OR). (See also Figure 3.2)

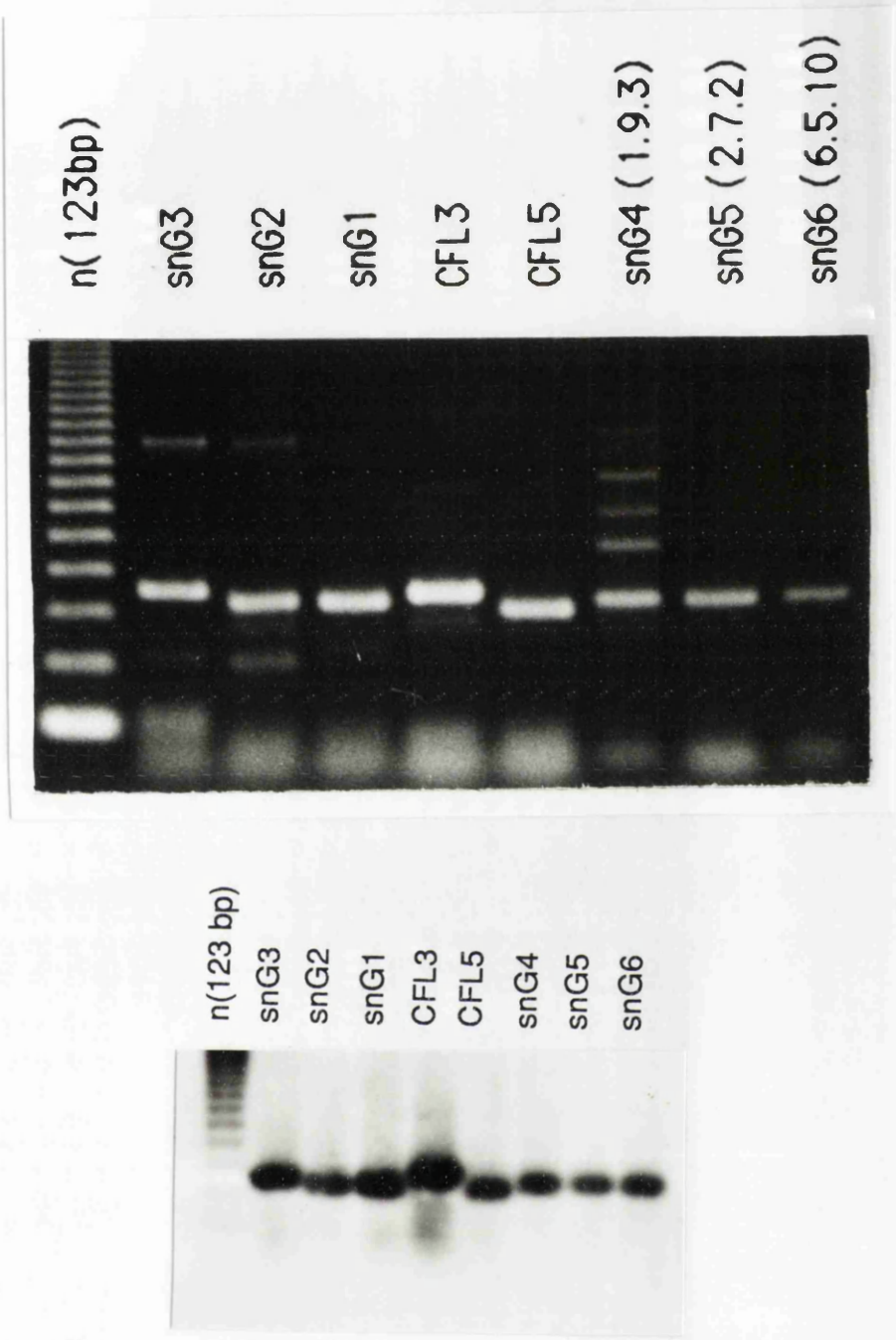


Figure 3.15. Analysis of DNA prepared from *sn* males. snG1-3 were produced by a traditional P/M dysgenic cross (By Dr Kim Kaiser); snG4-6 were produced by the dysgenic cross of Robertson et al., 1988. DNAs were amplified with P_{31} and snA. Only the predominant amplification products hybridise with pSn9. (*Upper*) Ethidium bromide-stained gel, 1.5% agarose. (*Lower*) Autoradiograph.

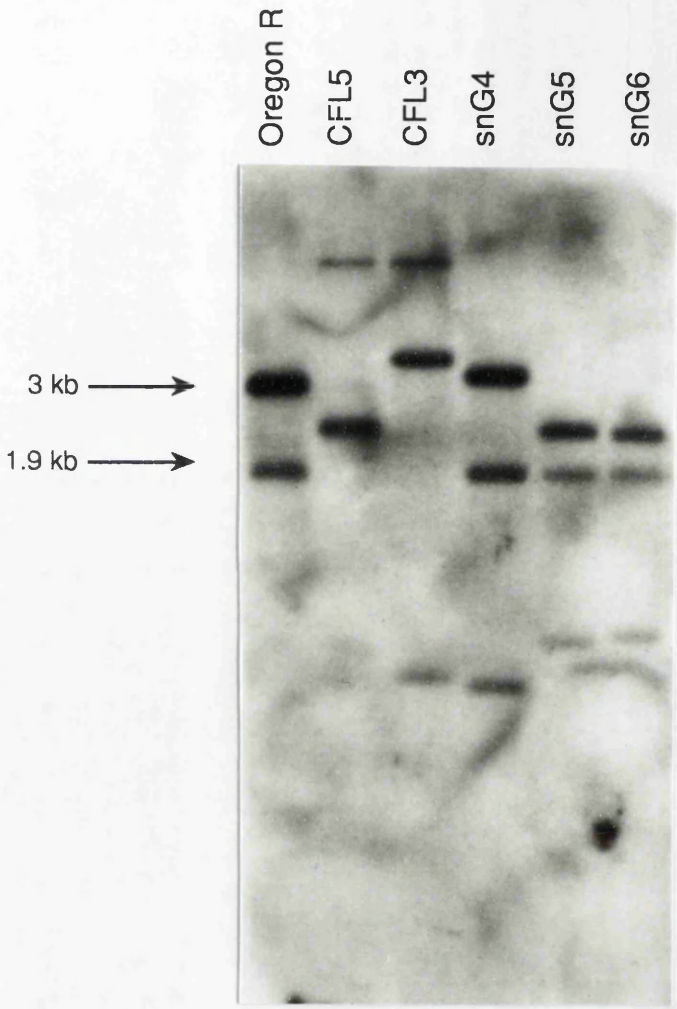


Figure 3.16. Restriction fragment length polymorphisms at the *sn* gene in the mutants snG4-6, wild-type Oregon R(OR), CFL5 and CFL3. DNA from *sn* males was digested with *Eco*RI and *Hin*dIII, separated in a 1% agarose gel, blotted, and probed with pSn9. The most 5' *Eco*RI site of figure 3.3 is not present in CFL3 and CFL5 strains.

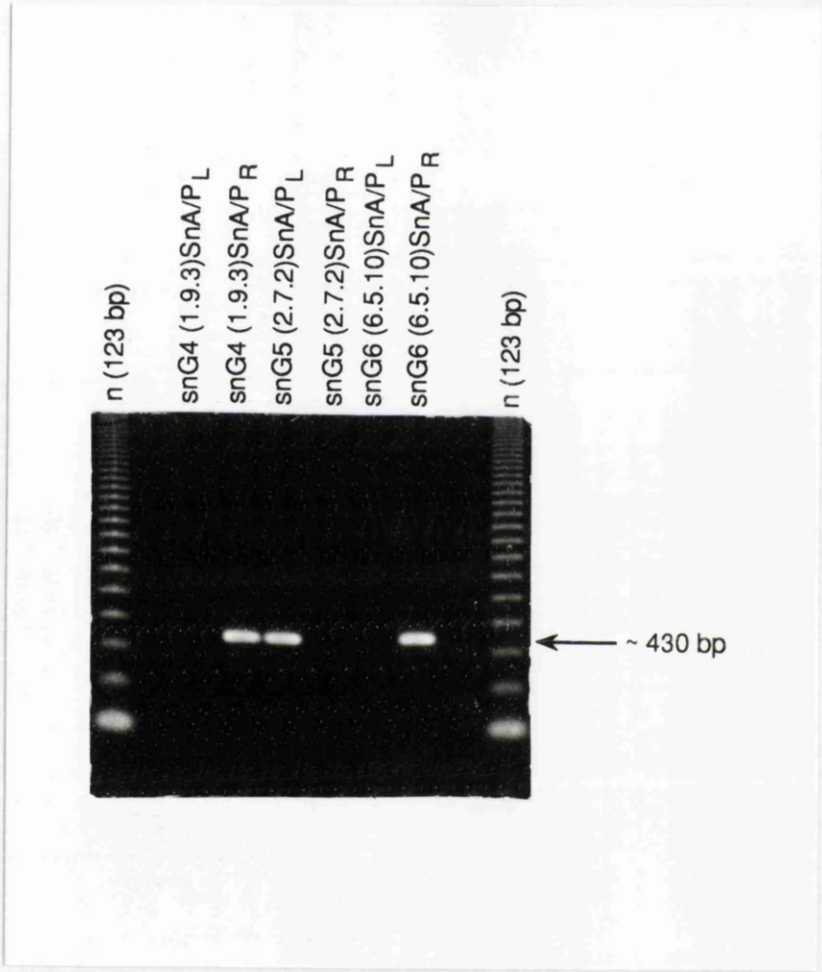


Figure 3.17. P-element-dependent amplification of the *sn* region in the mutants snG4-6. Amplification was with snA and either P_L or P_R primers individually. n(123 bp) corresponds to 123 bp DNA size marker (BRL). Ethidium bromide stained gel, 1.5% agarose.

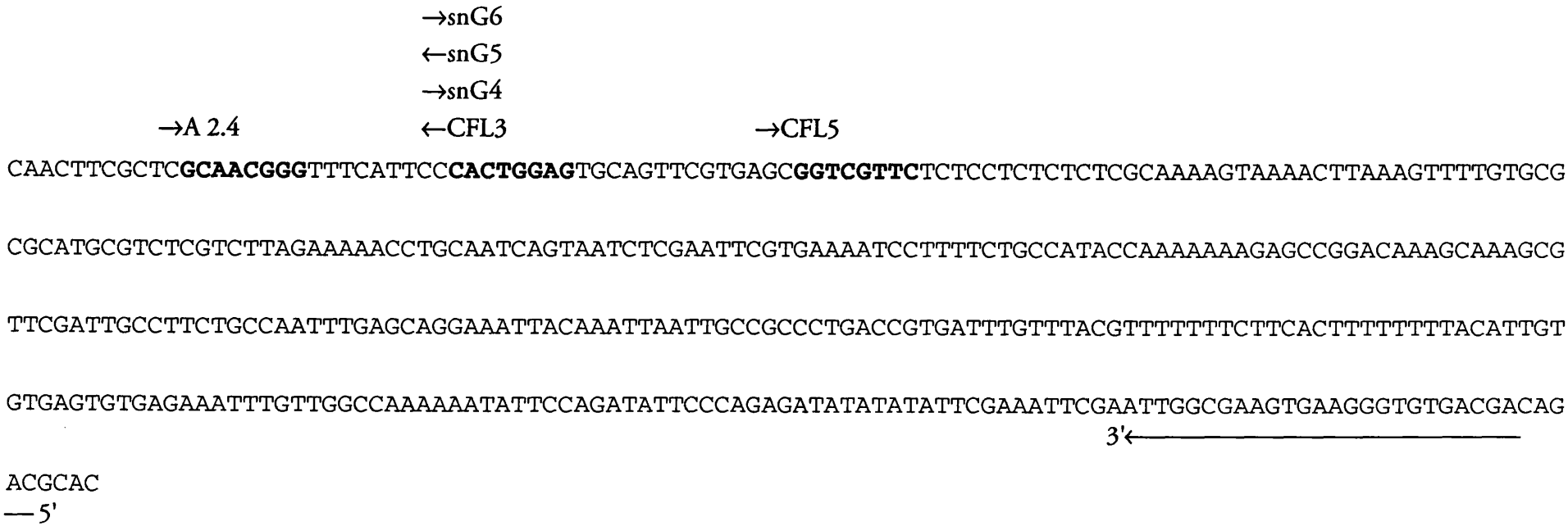


Figure 3.18. P-element insertions at the *singed* gene. The 8 bp duplication found at the ends of inserted P-elements is shown in bold. The orientation of the P-element associated with each mutation is shown. The arrowhead (→) corresponds to the right end of the P-element, which is 3' with respect to P-element transcription. Data for *sn* alleles A2.4, CFL3 and CFL5 taken from Roiha et al., 1988. Sequences of exon 1 of the *sn* locus are shown above. The DNA sequence has been published in the EMBL database (Heidelberg) under accession No. X17548. The position of the snA primer is shown as a large arrow (3'←——— 5').

elements have inserted at the same nucleotide position in independent events, but not all in the same orientation (Figure 3.18).

3.5 Long Distance Amplification

A preliminary experiment was carried out to determine the potential range of template amplification. Nucleotide sequence of the *sn* gene (Jamie Paterson and Kevin O'Hare, pers. comm.) was used to design an appropriate primer, snB, derived from the sequence beginning 11 bp downstream of the *Hin*dIII site of figure 3.3. The position of snB is approximately 2.7 kb away from the snA primer. PCR conditions were as described in section 2.3.3, with the exception of the extension time which was increased to 15 minutes. CFL3, CFL5 *sn* mutant and wild-type Oregon R DNAs were amplified with a combination of the appropriate primer pairs (P_{31}, snA and snB). From figure 3.19 it is clear, under these conditions, that amplification can occur between snA and snB with a wild-type template, but that amplification does not occur between snA and snB with either CFL3 or CFL5 templates. This may reflect the size constraints imposed by the PCR conditions used in this experiment as the P-element insertions present at the *sn* gene in CFL3 and CFL5 are 2.9 kb and 1.3 kb respectively (Roiha et al., 1988), thus making the anticipated amplification products > 4.0 kb in length. Amplification also occurs with P_{31} and snB with either CFL3 or CFL5 templates, producing amplification products of the anticipated size of approximately 2.3 kb. It would therefore be possible to detect a P-element insertion within about 2.5 kb of any given gene-specific primer.

Obviously the choice of primers is critically determined by the availability of appropriate sequence information. This is an extremely important consideration when designing primers for the "site-selected" mutagenesis of any cloned gene of interest. Ideally, a range of gene-specific primers would be used to cover all possible eventualities, but even with a bare minimum of sequence, it is possible to cover up to 5 kb of genomic sequence with two gene-specific primers.

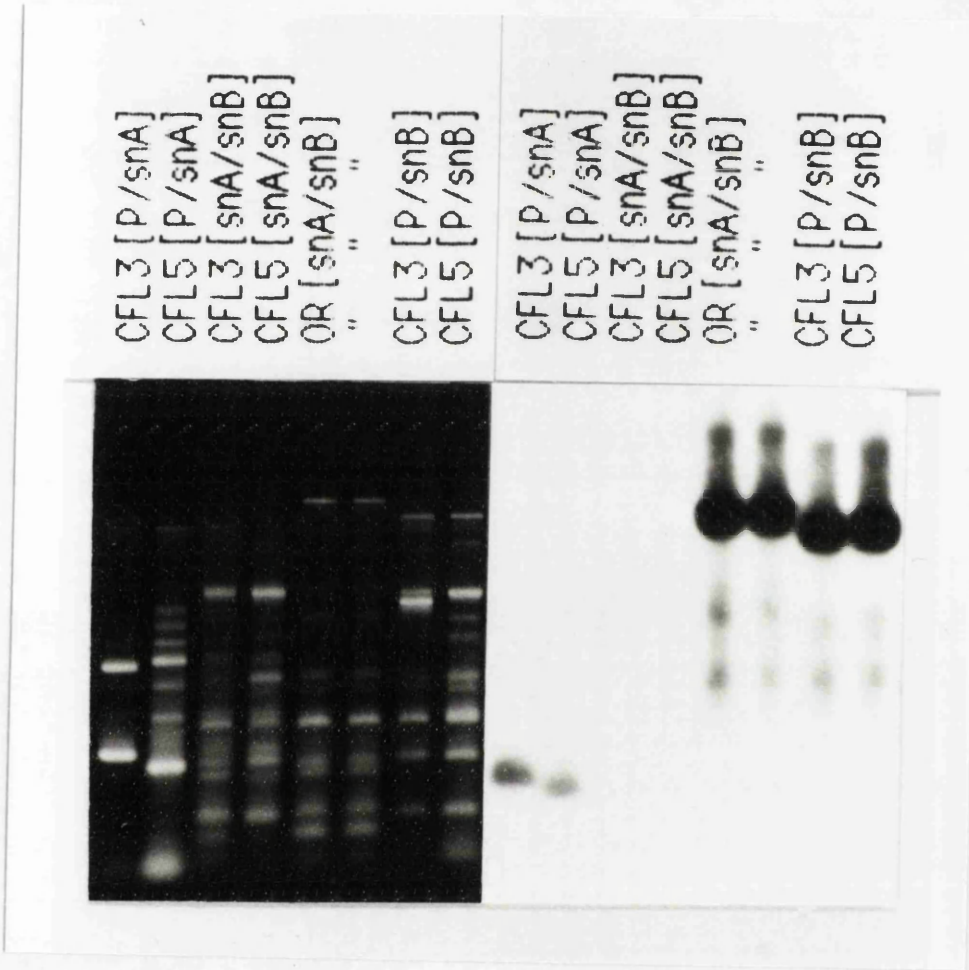


Figure 3.19. Long distance amplification. CFL3, CFL5 *sn* mutant and wild-type Oregon R (OR) DNAs were amplified with a combination of the appropriate primer pairs (P_{31}, snA and snB). PCR conditions were as described in section 2.3.3, with the exception of the extension time, which was increased to 15 minutes. (*Left*) Ethidium bromide-stained gel, 1.5% agarose. (*Right*) Autoradiograph, hybridised with pSn9.

3.6 Discussion

Presented in this chapter is the theory and demonstration of a "reverse genetics" approach termed "site-selected" transposon mutagenesis which allows the detection and isolation of P-element transposon insertions into or near a cloned gene of interest. Very little sequence is needed to design a gene specific primer and if more sequence information is available, a whole range of gene-specific primers can be designed for the cloned gene of interest. So for any gene appropriate primers could be designed to regions that are probable sites of P-element insertion, namely 5' regulatory regions (Engels, 1989). Moreover, primers from several different genes can be used to screen the same mutagenised population for insertions into any of the genes. Frequencies of P-element mutagenesis are non random (Kidwell, 1986; 1987; Engels, 1989) as the frequency of mutation of different loci varies enormously, from greater than 10^{-3} for the *singed* locus ("hot spot") to less than 10^{-6} for the *alcohol dehydrogenase* gene ("cold spot"). However, by applying the dysgenic cross of Robertson et al., 1988, approximately 10^4 mutagenised flies would be required for there to be a P-element inserted on average every 1 kilobase along the *Drosophila* genome. Since it would be permissible to screen 10^5 or more mutagenised flies by this method, one could be assured of success for almost any gene. Egg DNA saved from past experiments can be used for feasibility studies for mutating other genes of interest which would give an indication of the size of screen required. This method also allows the detection of "silent" insertions or those that confer phenotypes too subtle or esoteric to score by conventional screening strategies based on phenotypic criteria. Silent insertions close to the gene of interest may not be of immediate use, but remobilisation of a P-element is frequently associated with the deletion of flanking sequences (Engels, 1989) or local jumping to nearby regions of the genome (Tower et al., 1993; Zhang and Spradling, 1993). Such strategies can be used to generate lesions or new insertions that have phenotypic outcomes (Kaiser 1990; Pereira et al., 1992; Segalat et al., 1992; Hamilton et al., 1993; Littleton et al., 1993).

Since the frequency of P-element insertion into *Drosophila* genes varies considerably from gene to gene, with some genes proving totally resistant to P-element insertion (Kidwell, 1987; Engels, 1989), an alternative transposon with a different insertion specificity may be preferable. Milligan and Kaiser, 1993, using a model system have investigated the suitability of a retrotransposon, the *I* element (Finnegan, 1989), for "site-selected" mutagenesis. Their target was *white*, a known target for *I* element insertions, several *I*-induced alleles of which have been described previously. *I* elements transpose in the female progeny of "inducer" males (having functional *I* elements) and "reactive" females (lacking functional *I* elements). Amongst 10,000 mutagenised females screened in batches of 1,000, evidence was obtained for four independent insertions at *white*. *I* element biology is more complex than that of P-elements with factors like age and genetic background being significant determinants of transposition frequency. They also have a transposition mechanism precluding phenotypic reversion, as well as imprecise excision events as discussed for P-elements. From this evidence *I* elements are clearly not the transposable element of choice, but they may be of use especially if they benefit from "engineered" changes analogous to the P-element. Other alternative transposons for "site-selected" mutagenesis with different insertional specificity could include the *hobo* and *mariner* elements (Blackman, et al., 1989; Lidholm et al., 1993; Smith et al., 1993). Thus, as PCR-based screens become increasingly common, it may become clear that no one mutagen is a reverse-genetic panacea.

Clearly, the technique is not restricted to *Drosophila* as mutations in cloned genes could be isolated in any organism in which known transposable elements are active, providing that the mutation rate is not prohibitively low and in which large numbers of independent populations of animals could be easily manipulated. In fact, a "site-selected" strategy has been successfully used to isolate Tc1 insertions within a *C.elegans* myosin light chain gene (Rushforth et al., 1993). Table 3.1 summarises the success of "site-selected" mutagenesis approaches in *Drosophila* and other organisms to date.

Given that PCR detection strategies involving gene-specific primers and gene-specific probes require separate PCR reactions and hybridisations for each target gene

Organism	Transposon	Target gene	References
Drosophila	P-element	*singed* (*sn*)	Kaiser and Goodwin, 1990
Drosophila	P-element	RI, regulatory subunit of the cAMP-dependent protein kinase (PKA)	see chapter 4
Drosophila	P-element	ductin	see chapter 7
Drosophila	P-element	72H5 (adult compund eye cDNA)	Ballinger and Benzer, 1989
Drosophila	P-element	DCO, catalytic subunit of the cAMP-Dependent protein kinase (PKA)	Lineruth et al., 1992
Drosophila	P-element	G_i-like α subunit	Lineruth et al., 1992
Drosophila	P-element	G_o-like α subunit	Lineruth et al., 1992
Drosophila	P-element	Microtubule-associated Protein 205K	Pereira et al., 1992
Drosophila	P-element	*Pourquoi-pas?*	Segalat et al., 1992
Drosophila	I factor	*white* (*w*)	Milligan and Kaiser, 1993
C.elegans	Tc1	Myosin Light chain	Rushforth et al., 1993

Table 3.1 "Site-selected" transposon mutagenesis approaches in *Drosophila* and *Caenorhabditis elegans*

chosen, and the limitations of PCR over large distances, it has become apparent that a more consolidated approach may be needed. This would allow the simultaneous detection of insertions of many loci with a single PCR reaction. Sentry and Kaiser, (unpublished), have examined the feasibility of simultaneously targeting a number of different loci, without the need for gene-specific primers on a model system. Their method utilises an inverse PCR strategy (Ochman et al., 1988; Triglia et al., 1988; Silver, 1992) to amplify sequences flanking all new P-element insertions in a population of mutagenised individuals. This method involves circularisation of a restriction fragment containing sequences flanking the transposon, and then P-element specific primers oriented so that amplification proceeds "outward" from the transposon. Amplified DNA representing batches of mutagenised individuals can then be screened by hybridisation with a gene-specific probe. Alternatively, the amplified DNA can be labelled and hybridised to filters containing genomic or cDNA sequences of chosen genes. Such an approach would appear feasible, but certain factors must be taken into consideration for screening *en masse*, namely time. This procedure requires more manipulations, i.e. recircularisation, which relies heavily on the availability of desired restriction sites. To improve the chances of detection it may be necessary to generate DNA fragments with a variety of restriction enzymes. Ligation-mediated PCR (Loh et al., 1989; Mueller and Wold, 1989; Kaiser, 1990) is an approach which allows amplification of sequences which lie to one side of a region of known sequence. The specificity of both methods could be increased by using nested primers (first-round amplification products are further amplified using a primer pair that is capable of re-amplifying the first-round product), or in the case of Linker-mediated PCR by the use of vectorettes (Riley et al., 1990).

An extension of the "site-selected" mutagenesis strategy has been the mobilisation and detection of marked P-elements. Several such engineered elements have been described (O'Kane and Gehring, 1987; Bier et al., 1989). Although they are reasonably large and usually low in copy number, marked elements have been modified to include a dominant eye colour gene (w^+) which allows flies with insertions to be recognised (whose loss can be easily scored in an appropriate excision cross) and plasmid sequences that

facilitate the cloning of the flanking genomic DNA (see section 1.8.6, Chapter 1). Their major hindrance is that transposition rate is inversely proportional to element size (Spradling, 1986). Even strains with multiply marked elements are probably at least 100 fold less efficient than *Birm* -2 elements (Grell and Lindsley, pers. comm.). However, if one is screening a sufficiently large number of cloned genes, it may be possible to mutate a proportion of them. Hamilton et al., 1991 have described a method similar to "site-selected" mutagenesis that obviates the need for PCR by utilising marked P-elements that can plasmid rescue genomic sequences flanking the insertion site. By using a library of rescued plasmids from a population of P-*lacW* mutagenised flies as a hybridisation probe against an array of specific genomic or cDNA clones, they were able to test for insertions in or near to a gene of interest.

In the succeeding Chapters I shall describe the application of "site-selected" transposon mutagenesis in mutating the regulatory (RI) subunit of a cAMP-dependent protein kinase gene (PKA) with a view to testing the role of PKA in learning and memory in *Drosophila.*

Chapter 4

"Site-selected" transposon mutagenesis of the regulatory subunit (RI) of a cAMP-dependent protein kinase gene (PKA)

4.1 Introduction

A number of single-gene defects have been isolated that perturb associative and nonassociative learning processes (see section 1.3). The two most studied mutants are *dunce* (*dnc*), which disrupts the gene encoding the cAMP-specific phosphodiesterase II (Byers et al., 1981; Chen et al., 1986; Davis and Dauwalder, 1991; Kiger et al., 1981; Qiu et al., 1991) and *rutabaga* (*rut*), which disrupts the gene encoding the Ca^{2+}/Calmodulin-activated adenylate cyclase (Byers et al., 1981; Kiger et al., 1981; Levin et al., 1992; Livingstone et al., 1984). These findings implicated the cAMP signal transduction pathway in the neuromodulatory mechanisms underlying *Drosophila* learning and memory. This parallels the physiological findings from studies into learning and memory in the sea snail, *Aplysia californica* (see section 1.4 and chapter 6; Kandel and Schwartz, 1982).

Historically, the original set of learning mutants was isolated after chemical mutagenesis with ethyl methanesulfonate and screening for olfactory learning defects. This strategy has the advantage of offering a high rate of mutagenesis and the potential of mutating any gene required for learning that is nonessential. However, as a phenotype, behaviour can be quite variable and somewhat unreliable, making behavioural screening extremely slow and tedious (Tully et al., 1990; Dura et al., 1993). In addition, single base changes generated by chemical mutagenesis are difficult to map genetically, leaving the gene of interest difficult to identify by molecular means. In fact, of five mutants isolated by such screens, it is only *dunce* (*dnc*) (Byers et al., 1981; Davis and Davidson, 1984; Chen et al., 1986) and *rutabaga* (*rut*) (Levin et al., 1992) that have been cloned, primarily because other pleiotropic effects of the mutations were first identified. At present, many groups are using various approaches to aid in the genetic dissection of learning and memory in *Drosophila.* These include enhancer-trapping techniques to isolate new mutants based on behavioural or "anatomically preselected" phenotypes (Boynton and Tully, 1992; Han et al., 1992). "Dominant negative" mutations (Herskowitz, 1987) have been used successfully to study gene function, where one is

function, where one is devoid of an appropriate mutant (Drain et al., 1991
devoid of an appropriate mutant (Drain et al., 1991; Griffith et al., 1993).

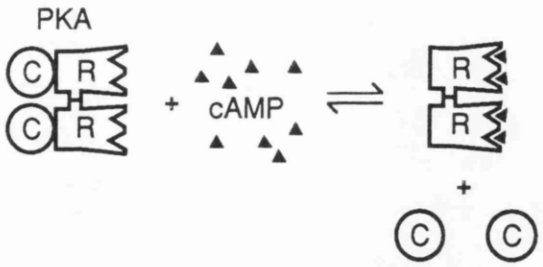
The detailed knowledge of the molecular components of the cAMP signal transduction pathway make it an ideal system for manipulation by reverse genetical approaches (see section 1.6; Sentry et al., 1993a). However, several important pieces of information are missing from present molecular models of learning (Dudai, 1989). For example, the identities of the relevant transmitters and receptors involved. In addition, the cAMP signal transduction cascade is only one of several pathways activated by transmitters, and the same transmitter often interacts with different receptors, coupled to different second-messenger pathways (Nestler and Greengard, 1984). Thus, although the cAMP signal transduction pathway is critical to learning processes, it is probable that other signal transduction pathways may be involved. Notably, the Ca^{2+}/calmodulin signal transduction pathway may be involved in neuronal plasticity (Nairn et al., 1985; Schulman, 1988; Griffith et al., 1993). In *Drosophila*, many of the pertinent genes have been cloned via homology, with no reference to phenotype (Buchner, 1991), examples include several classes of G proteins, a cAMP-dependent protein kinase gene and a Ca^{2+}/calmodulin protein kinase gene (Foster et al., 1988; Kalderon and Rubin, 1988; Hurley et al., 1990; Ohsako et al., 1993).

A large number of physiological cellular responses to extracellular signals have been shown to employ the second messenger, cAMP. The rate of cAMP synthesis, catalysed by adenylate cyclase, is modulated via a stimulatory (G_S) or inhibitory (G_i) G-protein that itself responds to an activated transmembrane receptor (Gilman, 1984; Levitzki, 1988). The known intracellular targets of cAMP are PKA (cAMP-dependent protein Kinase) and cAMP-gated ion channels (Delgado et al., 1991). PKA, however, is almost always the effector molecule for cAMP (Kuo and Greengard, 1969; Edelman et al., 1987; Nakamura and Gold, 1987). PKA, in the absence of cAMP, exists in an inactive tetramer composed of two heterodimers, each containing a regulatory (R) and a catalytic (C) subunit (Taylor et al., 1990). At elevated levels, cAMP binds to the regulatory subunits causing release of the active catalytic subunits to phosphorylate target

proteins (Figure 4.1a; Taylor et al., 1990). There are a large number of known hormones, neurotransmitters, and local chemical mediators that can modify the activity of adenylate cyclase, and there are a large number of potential substrates that can be altered in their activity by cAMP-dependent protein kinase phosphorylation (Nestler and Greengard, 1984; Nestler et al., 1984; Nairn et al., 1985). The specificity of a given cellular response is determined by the selective expression of receptors and effectors in the responding cell, making this system extremely adaptable (Nestler and Greengard, 1984; Nestler et al., 1984; Nairn et al., 1985). PKA holoenzymes are classified, based mainly on their elution from DEAE columns and differences in the structure of their R subunits, as type I (containing regulatory subunit isoforms RIα and RIβ) and type II (containing regulatory subunit isoforms RIIα and RIIβ) cAMP-dependent kinases (Taylor et al., 1990; Figure 4.1b). The regulatory subunits are linked as homodimers which combine with either of the three distinct catalytic subunit isoforms (Cα, Cβ and Cγ) (Scott and Soderling, 1992). Vertebrate studies have demonstrated that kinase isoforms are expressed in a tissue-specific manner, and by distinct genes (McKnight, 1991). Notably, the β-isoforms of each subunit are enriched in mammalian neuronal tissue (Cadd et al., 1989).

Drosophila genes for catalytic and regulatory subunits have been cloned and characterised (Foster et al., 1984; Foster et al., 1988; Kalderon and Rubin, 1988). Protein studies have shown that, as for other invertebrates and vertebrates, there are two forms of *Drosophila* regulatory subunit (Foster et al., 1984). A type II subunit (RII) is found in homogenates from all tissues and at all developmental stages. A type I subunit (RI) is detected at much lower levels in larvae (2nd and 3rd instar) and pupae (early and middle), but not in embryos or whole adult homogenates. RI is the only regulatory subunit so far cloned, by homology to a bovine type I cDNA (Kalderon and Rubin, 1988), in *Drosophila.* The *Drosophila* RI gene is a single copy gene, as judged by low-stringency nucleic acid hybridisation, and lies on the third chromosome in polytene division 77F. The RI gene produces at least three distinct transcripts (Class I, II and IV), one of which, the Class I, would encode a full length protein. The other two transcripts

(A)



INACTIVE ACTIVE

$$4cAMP + R_2C_2 \rightleftharpoons R_2(cAMP)_4 + 2C$$

(B)

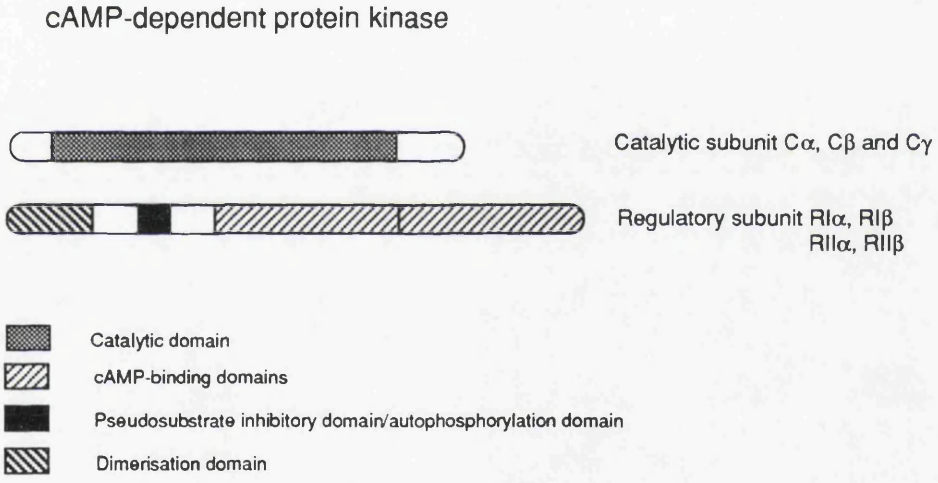


Figure 4.1. (a) Cyclic AMP activation of the cAMP-dependent protein kinase. Binding of cAMP to the regulatory (R) subunits causes dissociation of the catalytic (C) subunits. Only when dissociated from the R subunits is a C subunit an enzymatically active protein kinase. Each R subunit binds two cAMP molecules in a positive cooperative interaction. (b) The structural/functional domains of the catalytic and regulatory subunit of PKA. The PKA holoenzymes are subdivided into type I (regulatory subunit isoforms RIα and RIβ) and type II (regulatory subunit isoforms RIIα and RIIβ). From vertebrate studies, there are at least three catalytic subunits Cα, Cβ, and Cγ. Also indicated are pseudosubstrate domains and internal autophosphorylation sites within protein kinases. In the absence of cAMP, that binds to the regulatory domains, these domains interact with the active site to inhibit kinase activity (Taylor et al., 1990).

produce proteins that are truncated at the amino-terminus and lack a dimerisation domain (Figure 4.2; 4.3a). The deduced amino acid sequence of the full length protein has been compared with the murine RIα subunit, amino acid identity between the two sequences is 71% overall. In complete contrast to the protein studies, class I RNAs, the templates for the full length RI protein, are detected in all developmental stages. The remaining classes of RNAs are only detected in adults and all forms of RI gene transcript are enriched (at least fivefold) in head with respect to body. This suggests that the gene is expressed mainly in neural tissue. Functional differences between RI and RII are only partially understood. The most important distinction between them is that RII subunits are the subject of autophosphorylation and proteolytic modification, this is a characteristic of vertebrate and invertebrate subunits (Greenberg et al., 1987). There is evidence that these processes act to slow the rate of reassociation of RII with the catalytic subunit and may therefore lead to a prolonged increase of kinase activity (Müller and Spatz, 1989). A mechanism of this kind was originally proposed by Kandel and Schwartz (1982) as a molecular basis of classical conditioning in *Aplysia* and this system may represent part of a molecular correlate of memory in *Drosophila* (Müller and Spatz, 1989).

In a elegant study, Drain et al. (1991) provided compelling evidence that PKA has a role in associative learning and memory in *Drosophila*, by creating transgenic flies containing a peptide inhibitor of PKA or the pseudosubstrate inhibitory domain of the murine RII under control of a heat shock promoter they showed that olfactory learning was disrupted in these transgenic flies. Although this was the first demonstration of a direct role for PKA in *Drosophila* learning, mutational studies provide the only way to investigate the relative roles of different isoforms of the regulatory and catalytic subunits (see section 1.3.1; Skoulakis et al., 1993).

In this chapter, I have utilised the "site-selected" transposon mutagenesis strategy developed in chapter 3, to mutate the regulatory (RI) subunit of a cAMP-dependent protein kinase (PKA) with a view to testing the role of PKA in learning and memory in *Drosophila.*

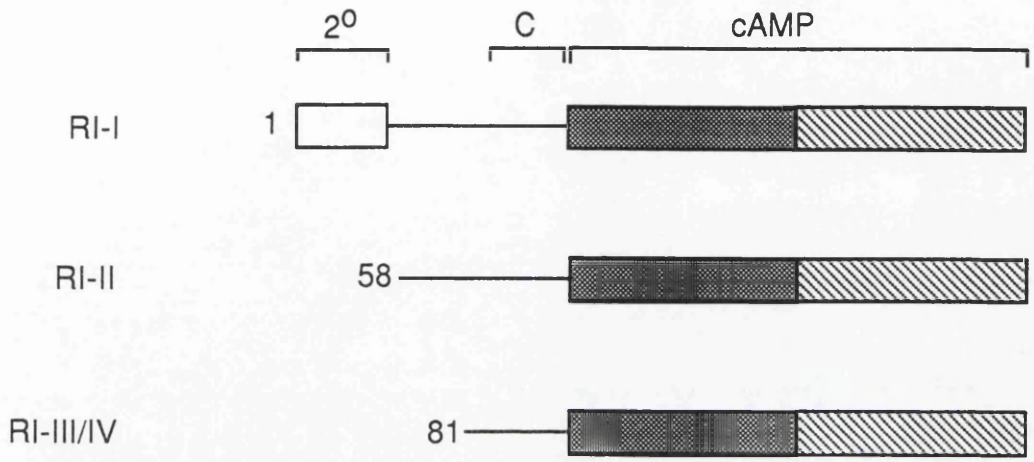


Figure 4.2. *Drosophila* type I regulatory polypeptides. Polypeptides theoretically encoded by the differently spliced RNA forms I-IV of the *Drosophila* RI gene are shown. The theoretical primary translation product of class I RNA is of approximately the same length as the mouse RIα protein, the product of class II RNA is missing residues 2-58 but is otherwise identical, and the product of class III and IV RNAs lack residues 2-81. By analogy with mammalian RII protein (Weldon and Taylor, 1985) both truncated *Drosophila* proteins (RI class II and RI class III, IV) would be expected to retain domains for binding of catalytic subunit (C) and cAMP (cAMP), but only the full-length analog would retain a dimerisation domain (2°). (From Kalderon and Rubin, 1988).

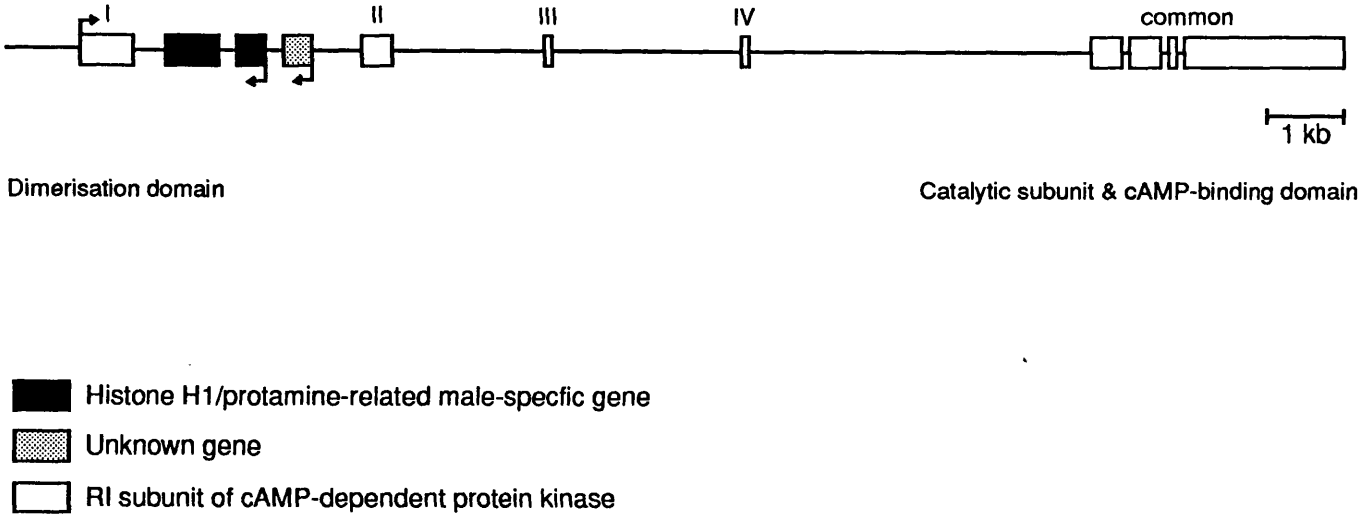


Figure 4.3a. Genomic organisation of the RI locus. A single *Drosophila* type I regulatory subunit gene, lying on the left arm of the third chromosome in polytene position 77F, is the source of at least three distinct transcripts originating from different promoters and spliced to a common body (C), see also figure 4.10. The RI locus also includes two intronic genes, a histone H1/protamine-related male-specific gene and a gene of unknown function. The diagram shows the map of the RI exons (I-IV, common): all exons of the RI gene spread over 16 kb were mapped by sequencing all genomic intron-exon junctions, as indicated by boxes. (Kalderon and Rubin, 1988).

100
ccggtctccgacggctggacaggatccagtgccatcaggattaccgcagggcaccagccatacaaaggatacgacggcgaggaagcacaggagcaggctg
200
cctatgaaacagcatttccttgccggtgacatgggggttcttttggacaaatcctcgccggtctgattatggtcccccaagatggctacattgtctgcat
300
tcgcatcgaaatcactgttgaagtgggacaactccgatcccactccaccattatcgctcaacatgacggaacggaaagtattctgcatattggtagcgta
400
gctactgccctccagttggaatttctgggtactcccggaaggttttcgagtcacaatcctggtggccgtgggagatgctctcgtatgtggtgatttcagg
500
gctagagtgttcggcgggatgcgtatcctttgggcttggagctgctccggcggactgggctgatgagcttgcttaagtttctgttgcaatttttgtcgat
600
gattggcggcgttgtggatctcaatttcctcgtcggattcagaatcgctcattgcctgacttagtggagcatagatgccctggtggccagtgggggcaa
700
taattcggagtgcagcattttagctgtcaaaacagagaacaagtccggaaaaatcaataccaaccggacaaaaccaatggtaaatagtccgaaaaagagg
800
gaaatcccaagtaaacccacctccgttgattggctcaaacacgctttgacatctaacatccacattgctactgcttttatctttattggattaggcgaaa
900
cattaattccaacgaacgaaaacaccctagaaaaagctcttcttaccgcaatttaaattcggtcgattgttatgcacaaaagatgttatcgataagcagt
1000
****
gttgcgcaaagtgaaacctatcgatatgaacgtgaccaAATCAGACGCTCACCACTGCATGCAGCCCAAAAAAAAACAACAACGTTTGACGTGCGTGAAAA
1100
TTGGTTAAGTTTTATGAAAATAACTTAGTTAACTGACTTTCCGACAGCCATCGATCAATCCATATGCTGCGGGGGAGTGCGGGGCTAAGGGCATTAATCA
1200
TTAGGACATTGCAAAGAGGTCCGGAAACGTAAATTTTGTCAAGTGAAAACGGGGTTGTGCTGTGTGTGTGGAAAACGGAACAAAACGGTGCCAAAAACGC
1300
AAGGAAAAGCTGCGAAAGCGGTGCACAGCAAACGGGTTGATAAAGCAGCAGGCTTAGACAAACGCGGCAGTCCAAGATATAGCCAAATTATCAGTGCTTT
1400
TAACCGATTTGCCAGCGAAACAATAACCAACATTCAATAACCACCCACACAAACGTATTGTGTACATAATATATACATCCATACCTACATACGTATATTT
1500
TGCGTAGTGCGGCGCTTGAAAGCTGTTTCCCCATCACCATCTCATCATCACTGCCATTCATTGCAAACCCACTCTGGAATCCATCCCACCTCCACCGTAC
1600
CACATGTCCTACATGATGGCCAAAACGCTGGAGGAGCAGAGCCTGCGGGAGTGCGAACACTACATCCAGACGCATGGGATCCAGCGAGTCCTCAAGGACT
M S Y M M A K T L E E Q- S L R E C E H Y I Q T H G I Q R V L K D C
GCATCGTCCAGCTGTGCGTCTGCAGACCCGAGAATCCCGTGCAGTTCCTGCGACAGTACTTTCAGAAACTCGAGAGGgtgagtccaaaaccaaaattata
I V Q L C V C R P E N P V Q F L R Q Y F Q K L E R
aataata

Figure 4.3b. (see opposite) Sequence of the First exon (I; see figure 4.3a) of the *Drosophila* RI gene. Transcribed regions are represented by capital letters; putative initiator and upstream methionine codons are highlighted (Red) as are the optional splice junctions at nucleotides 982, 1048, and 1408 that are used to generate different forms of class I RNA (see Figure 4.10). Heterogeneous start sites for class I RNA are between 939 and 964 (blue) and were mapped by primer extension (Kalderon and Rubin, 1988). The translation product of class I RNA is represented in single-letter code underneath the DNA sequence. The pair of gene-specific primers (77F1 and 77F2) designed to screen the 5'-flanking region of the RI gene and the male-specific gene are shown underlined. The 8 bp duplication site found at both ends of P-element insertion is marked by *. (From Kalderon and Rubin, 1988; sequence from EMBL accession no. X16970)

4.2 "Site-selected" transposon mutagenesis of the RI gene

4.2.1 P-element mutagenesis and "selection" strategy

Random P-element transpositions were generated by a hybrid dysgenic cross as described in section 3.4.1 (Figure 3.9). Briefly, *Birm-2; ry*^{506} males were mated at 16°C with *w; Sb P* [*ry* + Δ2-3] /TM6 females. F1 *Sb* male progeny were mated to wild-type females at 18°C. With assistance from Dr Kim Kaiser, whose help greatly facilitated the genetics described in this chapter, we opted to modify the "sib-selection" procedure (section 3.4.2) previously used to test the F2 *Sb* + mutagenised female populations. Individual F2 *Sb* + females were introduced to several wild-type males and placed in single vials. The vials were arranged into 10 x 10 arrays of vials and stored in 10 x 10 metal trays (30 cm x 30 cm, Denley trays). Each vial was given a two letter code, 1 to 10, in one direction (representing rows) and A to J in the other (representing columns). Thus each tray of vials represented 100 mutagenised females, with each female having a unique code, defined by a row and column position. Each female was allowed to lay for several days, and then, along with the wild-type male, removed. DNA was isolated from each female and a proportion of the DNA preparation was pooled into batches representing a column (A to J) and a row (1 to 10). Thus each batch represented 10 mutagenised females. DNA from the column batches (A to J) was screened for the presence of a P-element insertion as described in the section below. When the presence of an insertion was detected, the corresponding row batches were tested. By cross-referencing, it was hoped that this procedure would help to eliminate false positives, since verification of the P-element insertion was dependent on two separate PCRs. Consequently, the female of interest could be detected by cross-referencing the two codes.

4.2.2 "Site-selection"

It is not clear what is the basis for the insertional specificity of P-elements, but one theory favoured by researchers (Tsubota et al., 1985; Kelley et al., 1987; Engels, 1989) is that P-element insertions have a tendency to lie near the transcriptional start sites of genes. When one observes individual P-element insertion sites that have been analysed at various loci, those at or near transcription start sites do seem to outnumber those in other areas (see section 4.2.3). With this in mind, the nucleotide sequence of the RI gene (EMBL accession no. X16970) was used to design a pair of gene-specific oligonucleotides (77F1 and 77F2) to screen both the 5'-flanking region of the RI gene and a male-specific gene (Isolated by Drs S. Russell and K. Kaiser and by Kalderon and Rubin, 1988), located within the largest intron of the RI gene (Figure 4.3a; 4.3b). These primers were used simultaneously with two P-element specific primers, P_L and P_R, based on sequence within the *cis* acting determinants of P-element transposition, present in the nonautonomous P-elements of *Birm-2*. Two P-element specific primers were used in preference to P_{31}, since empirical observations suggested that P_L and P_R used in combination produced less artefactual amplification than P_{31} (see section 3.3; Figure 3.5b). These primers also allow the orientation of a P-element insertion to be determined. P-element insertions in the RI gene were detected by PCR amplification with the appropriate primers using DNA isolated from mutagenised females.

4.2.3 Isolation of P-element Insertions in the 5'-flanking region of the RI gene

12,000 mutagenised females were screened according to the "selection" strategy described (see section 4.2.1). From these mutagenised individuals, as judged by ethidium bromide stained gels and hybridisation using a ^{32}P-labeled RI genomic clone (λgS8), two independent insertions in the 5'-flanking region of the RI gene were recovered (*RI*^{7I5} and *RI*^{11D4}). No insertions were detected in the male-specific gene that resides in the largest RI intron. An example of one of these insertions is shown in Figure 4.4, for *RI*

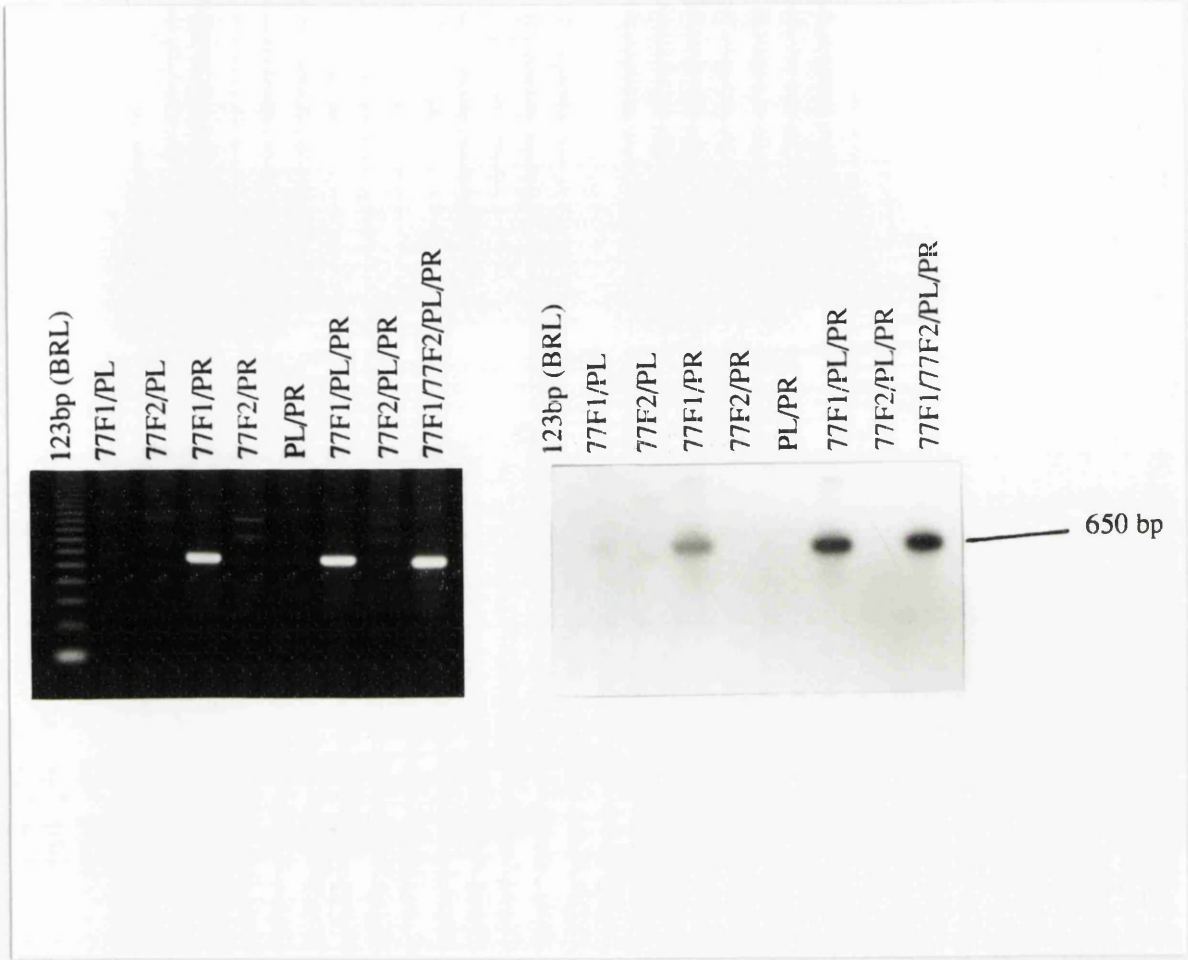


Figure 4.4. 12,000 F2 mutagenised females were screened as described, section 4.2.1. Four PCR primers were used simultaneously: two gene-specific primers (77F1 and 77F2) and two P-element specific primers (P_L and P_R). Two lines (*RI* ^{7I5} and *RI* ^{11D4}) gave rise to PCR products that hybridised with an RI genomic clone, λgS8. This figure illustrates one of these lines, *RI* ^{7I5} where amplification was dependent upon just one gene-specific primer (77F1) and one P-element specific primer (P_R). This was also the case for line, *RI* ^{11D4}. (*Left*) Ethidium bromide-stained gel, 1.5% agarose. (*Right*) Autoradiograph. 123 bp (BRL) corresponds to 123 bp DNA size marker.

^{7I5}, amplification of an ~650 bp product was dependent upon just one gene specific primer (77F1) and one P-element primer (P_R). This was also the case for *RI* ^{11D4}. To test for possible mutant phenotypes, both lines heterozygous for the P-element insertions were crossed with an appropriate third chromosome balancer (*w*; TM3, *Sb*, *Ser*, *e*/TM6B, *Tb*, *Hu*, *e*). Flies made homozygous for chromosomes carrying insertions *RI* ^{7I5} and *RI* ^{11D4} were viable and fertile, presenting no obvious phenotype.

DNA from the strains used in this study and the RI homozygous insertion lines was digested with *Bam* HI (does not cut within P-element sequence), separated in a agarose gel, blotted and probed with λgS8. Southern blot analysis of the two lines homozygous for the insertion shows disruption of the RI locus having occurred within the 1.5-kb *Bam* HI fragment that spans the insertion sites (Figure 4.5). The wild-type 1.5-kb *Bam* HI fragment was increased in size, shifting from 1.5-kb to 2.6-kb. Previous studies have determined that a region of DNA that includes an additional copy of the male-specific gene and the first exon of the RI gene is found on the Y chromosome (Kalderon and Rubin, 1988; Russell and Kaiser, 1993). Hybridisation to these Y chromosome sequences can be seen in Figure 4.5.

To determine the exact location of these two insertions, the 650 bp PCR products were recovered from an agarose gel and directly sequenced. The sequence of one of the PCR products showing the boundary between the P-element and the RI gene can be seen in Figure 4.6. The sequence boundary at both ends of the P-element insertions was determined (Figure 4.7). Both insertions have occurred in exactly the same position, within a ~26 bp region that contains heterogeneous start sites for transcripts encoding the full-length RI gene product (Figure 4.3b).

What is the basis of the insertional specificity of P-elements? One important factor may be the 8 bp target sequence which is duplicated upon insertion. O'Hare and Rubin, 1983, examined the sequences flanking 18 P-element insertions and found a consensus sequence: GGCCAGAC. The "hot spot" sites at *singed* (Roiha et al., 1988) and *Notch* (Kelley et al., 1987) match this consensus in at least 4 of 8 bp, and the *white* (O'Hare and Rubin, 1983) hot spot matches it perfectly. However, there are several

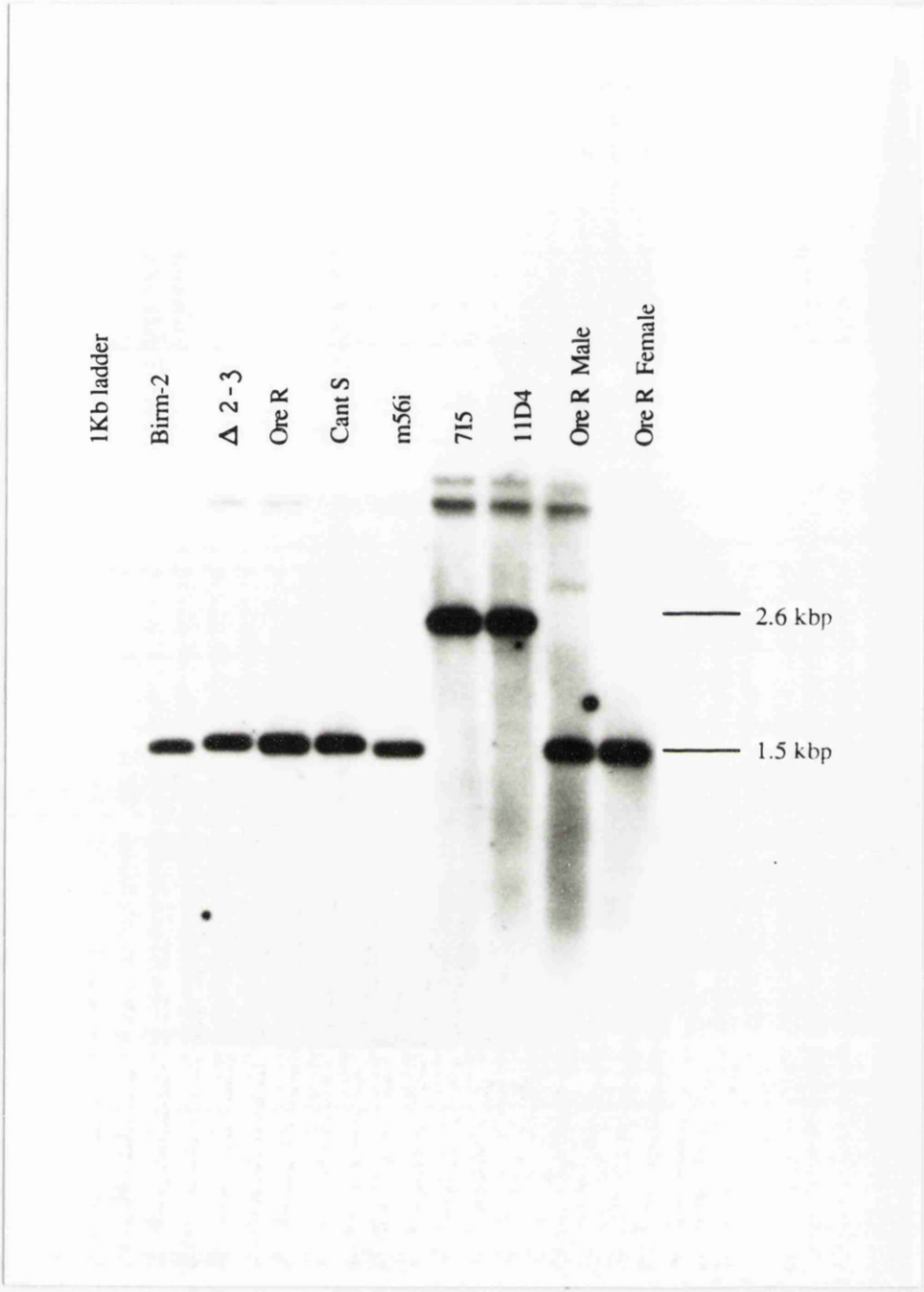


Figure 4.5. Restriction fragment length polymorphisms at the RI gene in the homozygous mutants *RI*^{715}, *RI*^{11D4}, and from the *Drosophila* strains used in the course of this mutagenesis. Genomic DNA was cleaved with *Bam*HI, separated in a 0.8% agarose gel, blotted, and probed with an RI genomic clone, λgS8.

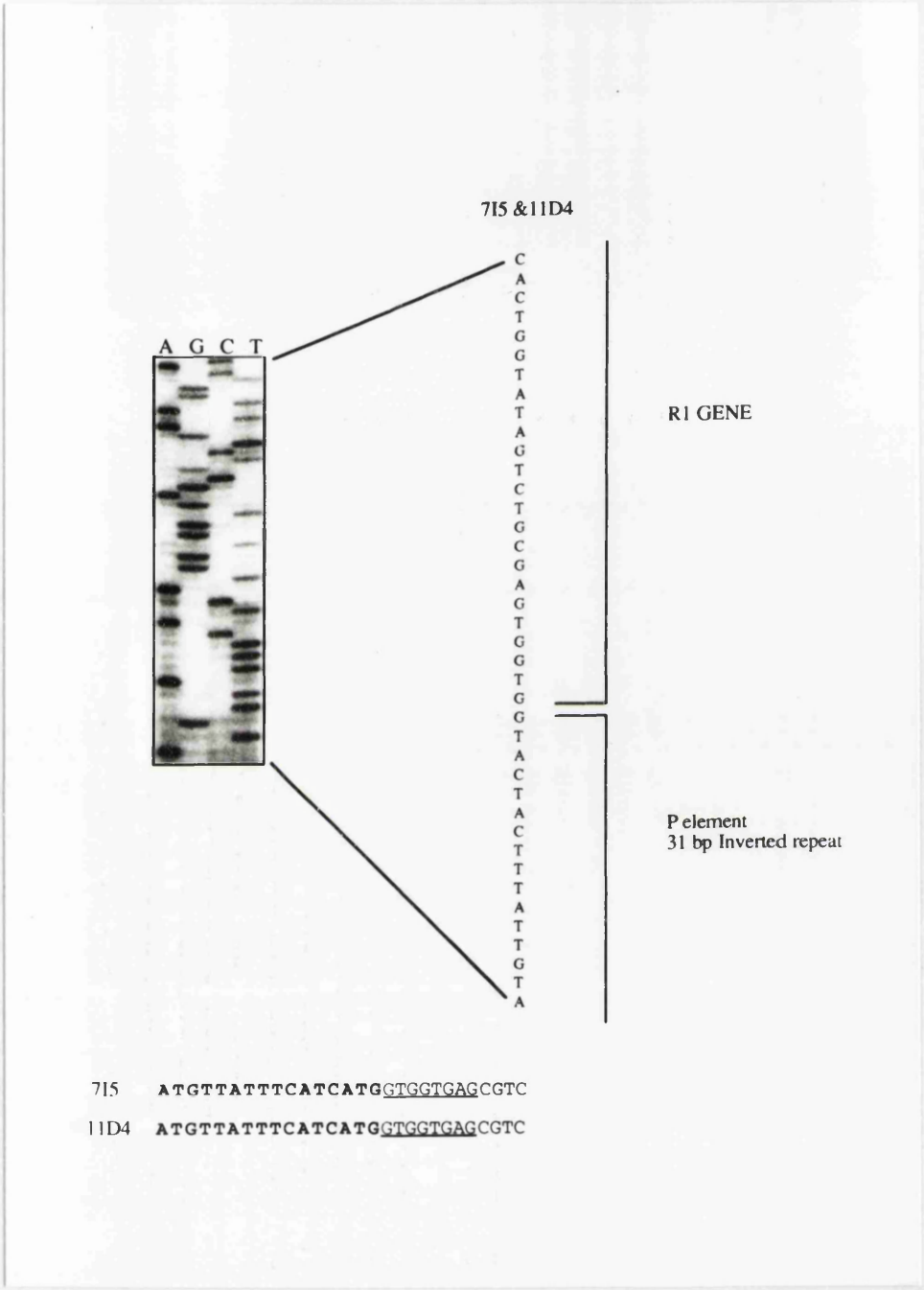


Figure 4.6. Direct DNA sequencing of the PCR product of *RI* ^{715} reveals the boundary between the P-element and the RI gene. The insertion of *RI* ^{11D4} is at the same position (data not shown).

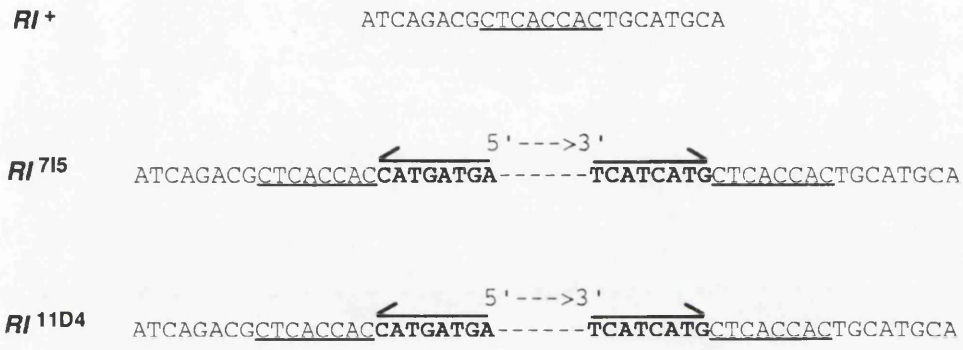


Figure 4.7. DNA sequence changes upon insertion of P-elements in the RI gene. P-element sequences are in bold face, and the 8 bp duplication found at the ends of inserted P-elements is underlined. 5'--->3' corresponds to the direction of P-element transcription. Sequences of the wild-type RI (*RI*+) locus are shown above. The RI genomic sequence has been published in the EMBL database (Heidelberg) under accession No. X16970.

non-hot spot sites at both *singed* and *Notch* that match the consensus better than the hot spots, indicating that the preference for this consensus is weak. This is also true of the RI insertions detected in this screen. The 8 bp duplication site: CTCACCAC matches the consensus in only 3 bp. An alternative suggestion (Tsubota et al., 1985; Kelley et al., 1987) is that hot spots have a tendency to lie near the transcription start sites of genes. Generally, when looking at individual P-element insertion sites that have been analysed at various loci (Tsubota et al., 1985; Chia et al., 1986; Eissenberg et al., 1987; Kelley et al., 1987; Howes et al., 1988; Roiha et al., 1988), as opposed to just hot spots, those at or near transcription start sites seem to predominate over those in coding and other regions. Thus a P-element insertion site may be critically determined by chromatin structure rather than a specific consensus insertion sequence.

4.2.4 The insertion present in both lines is a defective P-element

Both *RI* ^{7I5} and *RI* ^{11D4} were isogenised with a wild-type (*C-S*) strain by extended out crossing to separate the P-element insertion from any other elements on the chromosomes. The insertions were followed (for more than six generations) as amplification products, thus allowing free recombination to occur. The number of P-elements remaining in the recombinant chromosomes was determined by Southern blot analysis. DNA from *RI* ^{7I5}, *RI* ^{11D4} homozygotes and wild-type (*C-S*) was digested with *Bam* HI, separated in an agarose gel, blotted and probed with a P-element probe, pπ 25.7 WC. Both *RI* ^{7I5} and *RI* ^{11D4} probably contained only one P-element, at the RI locus (Figure 4.8a). By inference from Southern blot analysis of the two lines, the disruption at the RI gene had been caused by an insertion of an ~1 kb defective P-element (Figure 4.8a; Figure 4.5). To ascertain if they were independent transpositional events genomic DNA prepared from both strains was amplified using a single primer derived from the 31 bp inverted repeat present at both ends of all naturally occurring autonomous and non autonomous P-elements. This primer P_{31Inv} is orientated such that amplification proceeds into the P-element from each end. DNA prepared from both strains after

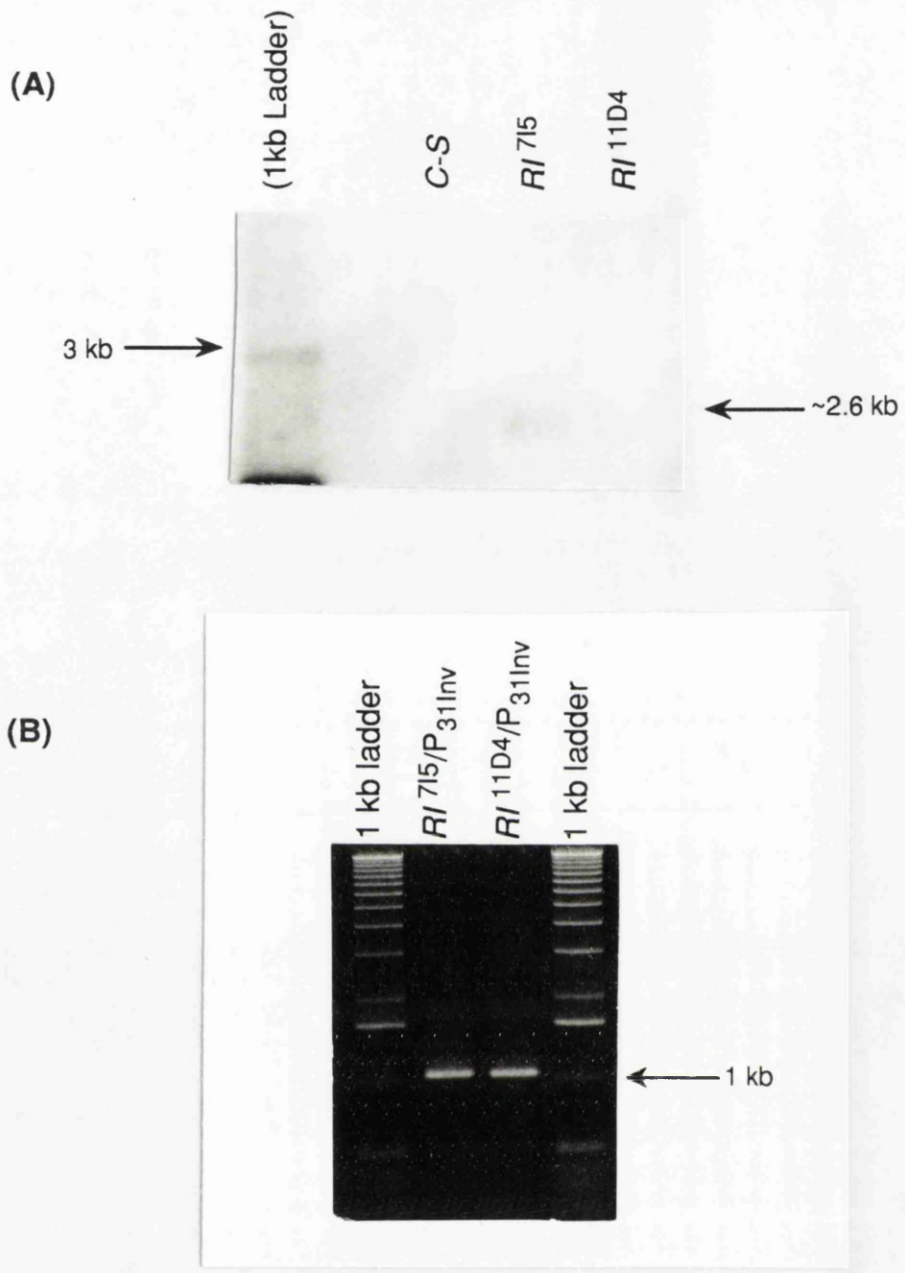


Figure 4.8.

(A) Genomic blot southern analysis of RI homozygous mutants, to determine the number of P-elements remaining in the recombinant chromosomes. Genomic DNA from *RI* ^{7I5}, *RI* ^{11D4} homozygotes and wild-type (*C-S*) was cleaved with *Bam*HI, separated in a 0.8% agarose gel, blotted and hybridised with a P-element specific probe, pπ 25.7 WC.

(B) Genomic DNA from *RI* ^{7I5} and *RI* ^{11D4} homozygotes was amplified with P_{31Inv}. Amplification reactions were separated on a 1.5% agarose gel stained with ethidium bromide. (1 kb Ladder) corresponds to 1 kb DNA size marker (BRL).

amplification with P_{31Inv}, produced a single PCR product of ~1 kb in length (Figure 4.8b). These PCR amplified P-elements were recovered from an agarose gel and directly sequenced using a range of primers corresponding to P-element sequences (a kind gift from K.O'Hare; O'Hare et al., 1992). Both were found to be a defective P-element derived from a 2.9 kb P-element (O'Hare and Rubin, 1983) by the internal deletion of 1848 bp between nucleotides 394-2241, (Figure 4.9) and both are in the same orientation with the proposed direction of transcription (O'Hare and Rubin, 1983; Karess and Rubin, 1984) being the same as that for the RI gene. Although the two mutant lines (*RI* ^{7I5} and *RI* ^{11D4}) were generated independently, they both have insertions identical in size, insertion site, and orientation and I consider them to be functionally equivalent.

4.3 Insertions in the RI gene disrupt expression of the longest RI transcript

Kalderon and Rubin, (1988) have previously determined that the *Drosophila* RI gene is the source of at least three distinct transcripts originating from different promoters and spliced to a common body (Figure 4.3a; 4.10a; 4.10b). Differential splicing of transcripts results in the generation of at least three putative RI polypeptides, two of which are truncated at the amino terminal relative to the largest, Class I, transcript. The Class I transcript, giving rise to a theoretically full length protein, is expressed at all stages of development and is enriched in adult head (Kalderon and Rubin, 1988).

The P-element associated with *RI* ^{7I5} and *RI* ^{11D4} has inserted in the heterogeneous transcription initiation sites for Class I mRNAs (Figure 4.3b; 4.10). Therefore, the introduction of a P-element within this site may affect gene expression, and ultimately, lead to a mutant phenotype. To explore the mechanism by which the insertion might alter gene expression, adult head poly(A)$^+$ RNA from wild-type (*RI*$^+$) and *RI* homozygous mutants (*RI* ^{7I5}, *RI* ^{11D4}) was hybridised as indicated to RI specific probes (the locations of which are shown in Figure 4.10c). Isolated RNAs were hybridised with a cDNA probe (pcD6BO RXba), which represents sequences found in

RI 7I5

```
 380         390         400
  *           *           *
AAAGTGATAGAGCCTGAACCAGAAAAGATAAA
|||||||||||||||||
AAAGTGATAGAGCCTGTAAAGATGATGAGCCT
                  ||||||||||||||||||
AAAACGAAAATAAAAGTAAAGATGATGAGCCT
      *           *           *
    2230        2240        2250
```

RI 11D4

```
 380         390         400
  *           *           *
AAAGTGATAGAGCCTGAACCAGAAAAGATAAA
|||||||||||||||||
AAAGTGATAGAGCCTGTAAAGATGATGAGCCT
                  ||||||||||||||||||
AAAACGAAAATAAAAGTAAAGATGATGAGCCT
      *           *           *
    2230        2240        2250
```

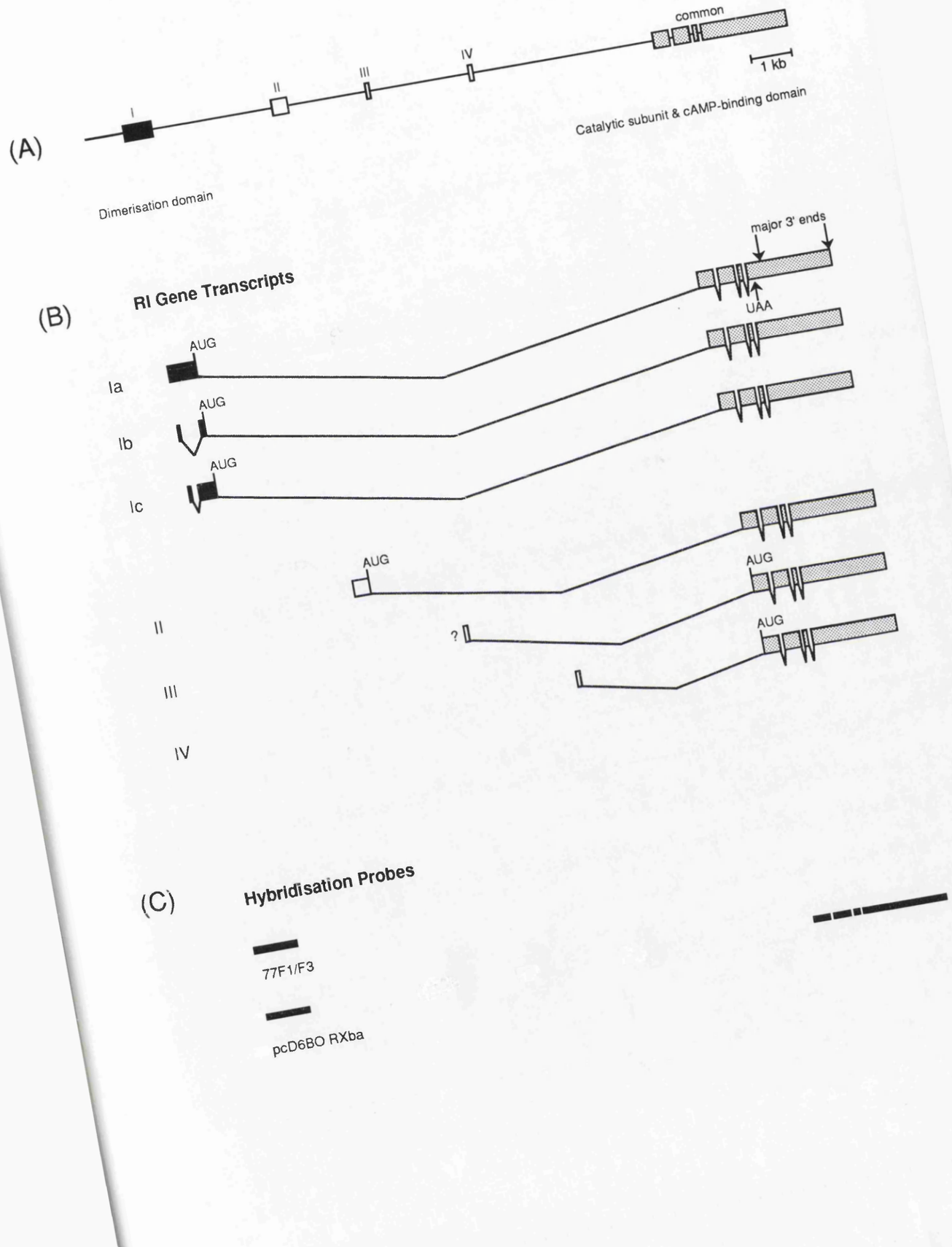
Figure 4.9. DNA sequences of internally deleted P-elements from *RI* ^{7I5} and *RI* ^{11D4} homozygous mutants. The sequences of internally deleted P-elements are shown for the position corresponding to the deletion of sequences from the intact 2.9 kb element (O'Hare and Rubin, 1983; accession number V01520). They are aligned with the sequence of the 2.9 kb element from both ends of the presumptive deletion (Full length 2.9 kb sequences are in blue case).

Figure 4.10. (see opposite page)

(A) Structural organisation of the *Drosophila* RI locus. Map of RI exons: four different exons I-IV, that may be spliced to a common region (common) of the RI gene, spread over 16 kb are shown. Between the splice donor specific to class I RNAs and the 5' end of class II RNAs, in the largest intron, are two genes, a male-specific gene (Histone HI/protamine-related) and an unknown gene (Figure 4.3a). (From Kalderon and Rubin, 1988).

(B) RI gene transcripts. The RI gene transcripts shown have been inferred from cDNA analysis, Northern analysis, and S1-nuclease protection experiments (Kalderon and Rubin, 1988). Multiple RNA species are derived from the RI gene, by differential splicing both upstream of and within the coding sequence of the gene and by the use of different polyadenylation sites. Class I, II, and IV RNA species have different 5' ends (5' end of class III has not been mapped). Heterogeneous start sites for class I RNAs span a region of 26 nucleotides. Each of these RNAs is spliced from a unique 5' exon to a common series of exons and may be polyadenylated at one of two major sites in the 3' UTR. Presumptive initiator codons (AUG) for polypeptides derived from class I-IV RNAs are shown. Two minor variants of class I RNA (giving rise to the same polypeptide) were identified from cDNAs (Ib and Ic) indicating splicing in the 5' UTR between a single donor and two alternative acceptors. The major form, Ia, is not spliced in this region. (From Kalderon and Rubin, 1988).

(C) The locations of sequences used as hybridisation probes for the RNA blots shown in Figure 4.11 are shown underneath the RI gene transcripts.


(A)
common
IV
III
II
I
1 kb
Catalytic subunit & cAMP-binding domain
Dimerisation domain
(B)
RI Gene Transcripts
major 3' ends
UAA
AUG
Ia
AUG
Ib
AUG
Ic
AUG
AUG
II
?
AUG
III
IV
(C)
Hybridisation Probes
77F1/F3
pcD6BO RXba

all 3 classes of RI transcript. The class I, II and IV RNAs (3.8 kb, 3.4 kb and 3.2 kb respectively in size) are present in all three lines tested (Figure 4.11). Both *RI* ^{7I5} and *RI* ^{11D4} have an extra transcript of approximately 4.8 kb in length. Figure 4.11, shows the results of hybridising the samples with a smaller probe, pBKS- 77FI/77F3, which spans the point of insertion (class I specific only). This probe hybridises to the 3.8 kb *RI*$^{+}$ RNA and the 4.8 kb and 3.8 kb *RI* ^{7I5} and *RI* ^{11D4} RNAs. Thus in both *RI* ^{7I5} and *RI* ^{11D4} a transcript of a discrete novel size accumulates that contains the sequences of wild-type RI transcript 3' to the site of insertion. Bearing in mind the class I RNAs are initiated from a 26 bp heterogeneous start site, I propose that the 4.8 kb transcript present in *RI* ^{7I5} and *RI* ^{11D4} is initiated upstream from the P-element insertion, or even from within the P-element, and that the 3.8 kb transcript present in *RI* ^{7I5} and *RI* ^{11D4} represents a transcript initiated downstream from the insertion. It may be significant that from the proposed direction of transcription of the P-element (O'Hare and Rubin, 1983; Karess and Rubin, 1984), the P-element in *RI* ^{7I5} and *RI* ^{11D4} could be transcribed in the same direction as the RI gene. Is the larger 4.8 kb RNA transcript homologous to both RI and P-element sequences? To test this hypothesis, the blot shown in Figure 4.11 was stripped, and probed with a P-element specific probe, pπ 25.7 WC. No hybridisation was detected when the filter was probed with the P-element sequences. I am at a loss to explain this, but further experiments, including RT-PCR, will be used to determine the exact nature of this 4.8 kb RNA transcript.

If this hypothesis proved to be correct one might predict that the incorporation of P-element sequences into the Class I transcript would interfere with translation. The residual P-element sequences in the mutant transcript introduce additional short reading frames upstream of the presumed RI translation start site. Although it is impossible to infer which mechanism is operating on the basis of the available data, eukaryotic translation models (Kozak, 1992) would predict that these new reading frames might interfere with translation initiation at the usual site. Secondary structure in the 5' leader region of the Class I transcript could interfere with the early phase of translation eg: binding or migration of a ribosome preinitiation complex.

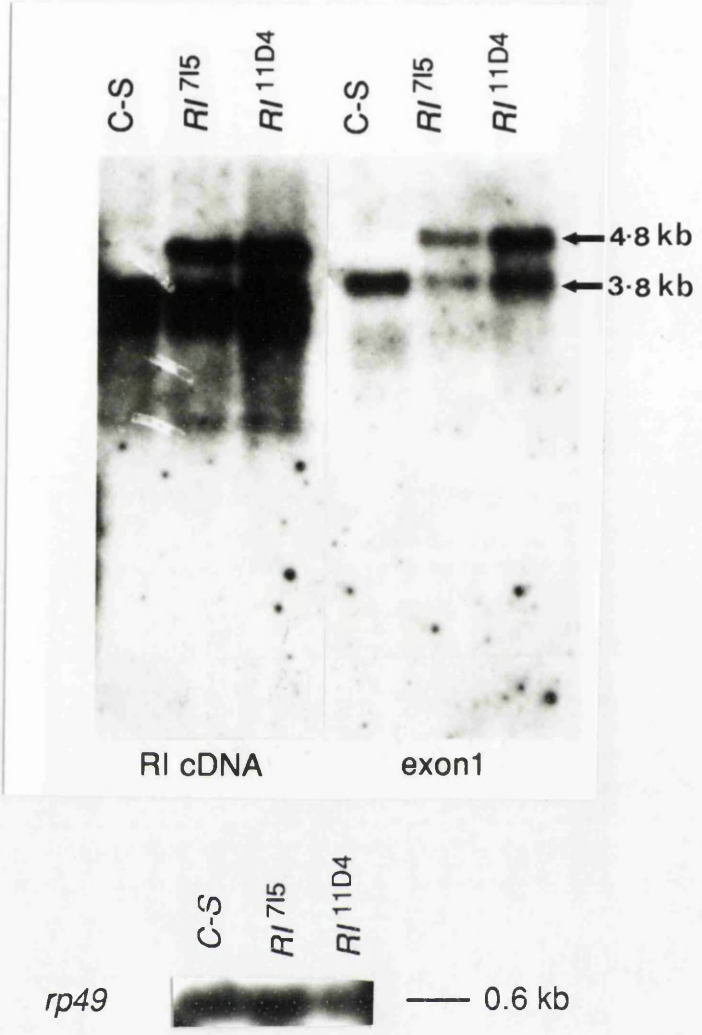
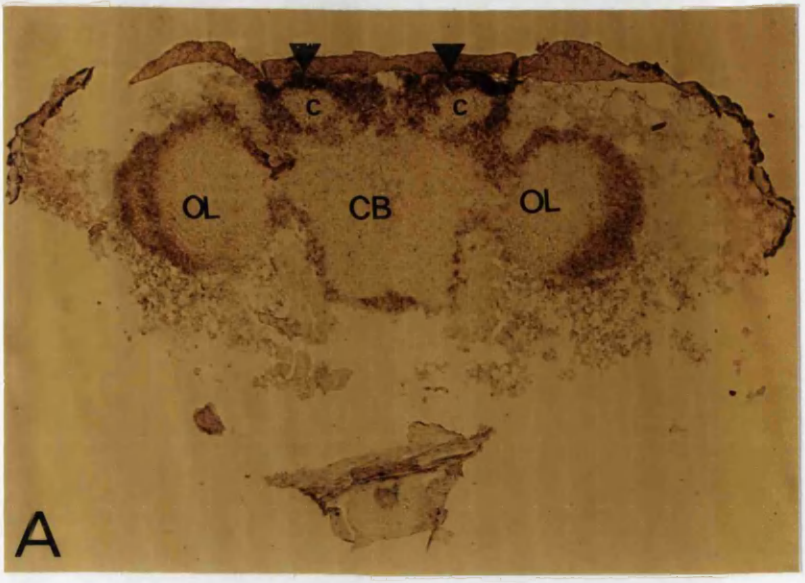


Figure 4.11. Northern analysis of wild-type and mutant RNAs. RI gene transcripts. RNA blots of adult head poly(A)$^+$ RNA (5µg per lane) were hybridised to the indicated probes, the locations of which are shown in Fig. 4.10. The probe used was specific to all RI transcripts (A) and in (B) to class I transcripts only. Subsequent hybridisation of these blots with a ribosomal protein gene probe, *rp49*, showed that all lanes have roughly equal amounts of intact RNA, of the size of *rp49* (0.6 kb).

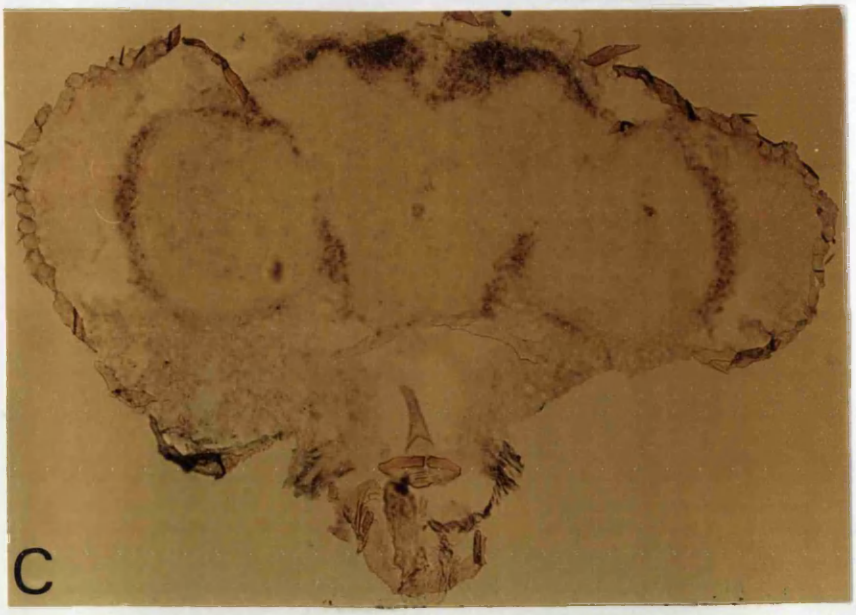
4.4 *In situ* hybridisation with RI probes

Genes central to learning and memory processes in *Drosophila* have been analysed by *in situ* hybridisation/immunohistochemistry and by the use of reporter gene constructs. Examples of these include *dnc* (Nighorn et al., 1991) and *rut* (Han et al., 1992) and *DCO*, the catalytic subunit of PKA (Skoulakis et al., 1993). All have elevated expression in a specific region of the brain, called the mushroom bodies (MB) (see section 1.3.2; Figure 1.3). Other lines of evidence have suggested that mushroom bodies are crucial for information processing and integration in insects. For example, two *Drosophila* mutants with altered mushroom body structure show disruption of olfactory conditioning (Heisenberg et al., 1985) and in the honeybee, local cooling of the mushroom bodies produces amnesia after training with olfactory cues (Erber et al., 1980). More recently, de Belle and Heisenberg (1993) have used a non-genetic approach to generate mushroom body-ablated *Drosophila*. Feeding first instar larvae (± 1 hour old) with hydroxyurea for 4 hours specifically kills the five pairs of cells which gives rise to mushroom body kenyon cells and a small population of cells in the antennal lobes. These MB-ablated flies are unable to perform in a classical conditioning paradigm.

Since it had not been determined previously (Kalderon and Rubin, 1988), I was particularily interested to determine the specific areas of the fly brain in which the RI gene was expressed. The spatial pattern of the RI gene was revealed by RNA *in situ* hybridisation with an RI cDNA probe (pcD6BO RXba) to frozen sections of wild-type heads (sections kindly provided by M. Yang). Single-stranded antisense and sense (control) DNA probes labeled with digoxigenin were generated by the polymerase chain reaction as described by Patel and Goodman (1992). The *Drosophila* major opsin gene was used as a tissue-specific control probe (pST41, a gift from S. Tomlinson; Figure 4.12). The highest level of hybridisation, with an antisense probe, was found in the cell body region immediately dorsal and posterior to the calyx neuropil at the mushroom body perikarya. The rest of the cortex showed a lower level of staining (Figure 4.12). No evidence was found for the presence of RI mRNA in neuropil structures (space in the central nervous system


C
C
OL
CB
OL
A


B


C

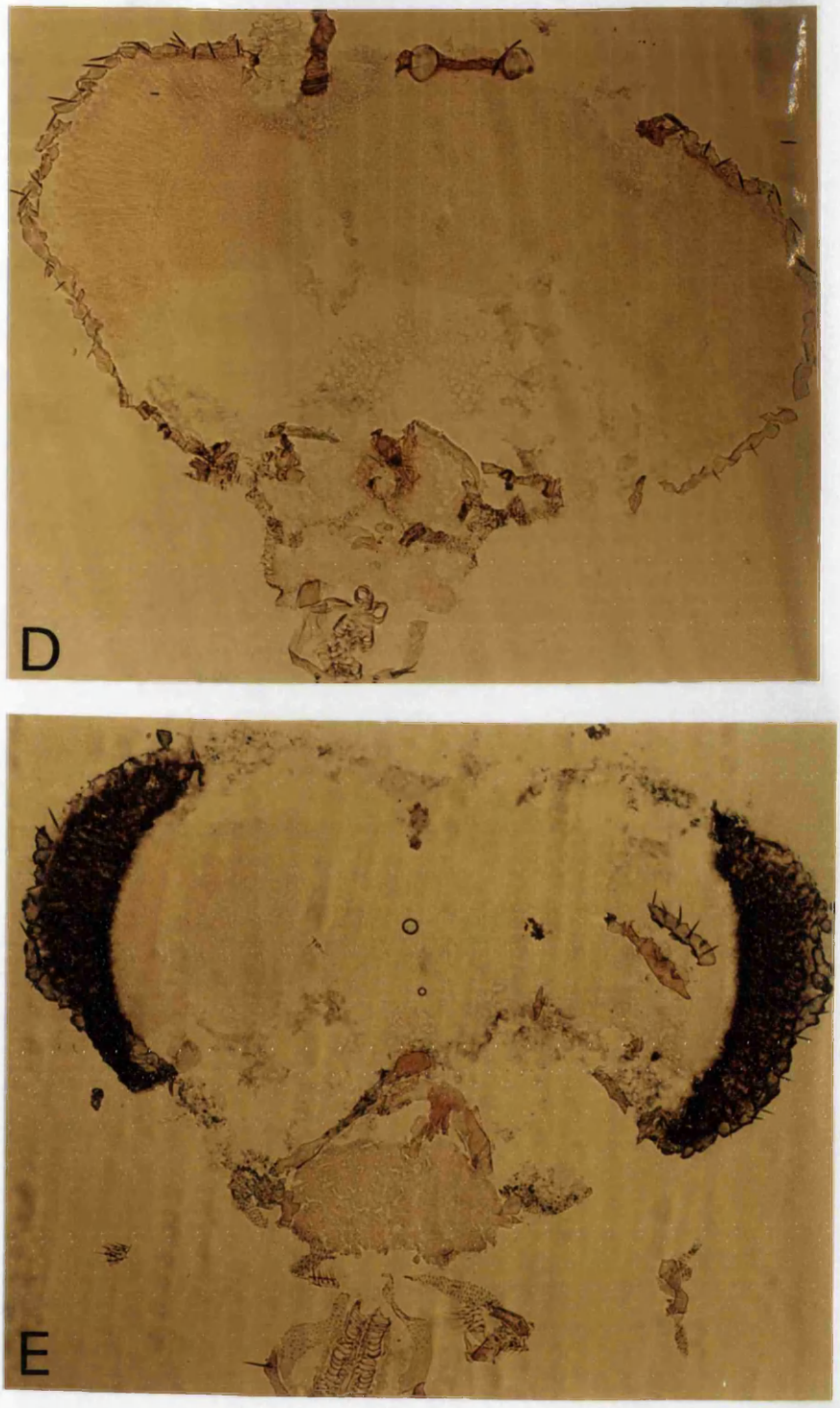


Figure 4.12. (see opposite page) *In situ* Hybridisation of the Adult Central Nervous system. RNA *in situ* hybridisation to a series of 12µm frontal sections at 200 X magnification. Dorsal is up in all sections. Hybridisation with an antisense probe to RI RNA applied to a frontal section at the level of the calyces(A-C) showing staining of cell bodies with staining of the cell bodies dorsal to the calyces (arrows). C, calyx; CB, central brain; OL, optic lobes. No staining was observed with an RI sense probe (D). (E) Hybridisation with an antisense probe to pST41 (control opsin).

occupied primarily by dendrites and axons) and no hybridisation was observed with a sense probe.

4.5 Generation of Precise and Imprecise excisions

Given that the insertion lines are homozygous viable, it would be of interest to determine whether the RI gene is entirely dispensable. The insertions isolated are within a ~ 26 bp region that contains heterogeneous start sites for transcripts encoding the full-length RI gene product and are ~ 550 bp upstream of the putative translation start site, although some forms of mRNA could use ATGs much closer to the start site (Figure 4.3; Figure 4.10; Kalderon and Rubin, 1988). P-element insertions in or near a gene of interest can be mobilised to recover new alleles of the gene through imprecise excision, chromosomal rearrangements, and other changes (see section 1.8.4), providing an important method for inducing targeted genetic changes. Briefly, the procedure is to screen for new variants in the progeny of dysgenic flies carrying the insertion. The resulting new alleles can be internal deletions of the P-element, deletions of flanking sequences (usually one-sided, ranging from a few bp to many kb in length), or secondary insertions. In the case of the RI gene, deletions that extend upstream would be expected to remove essential *cis*-acting control sequences, while deletions >550 bp in length will remove coding sequences. Thus a range of mutations, including true nulls, could be generated. Recently it has been discovered that P-elements jump at elevated frequencies into regions closely flanking their sites of origin (Tower et al., 1993; Zhang and Spradling, 1993). Peculiarly, this phenomenon appears to be limited to the female germ line. As many as 1% of progeny chromosomes can have new insertions within 200 kb of a donor element.

To test all these possibilities, a pilot experiment was initiated (this experiment is still in progress). Using a simple genetic scheme (Figure 4.13), males homozygous for the RI insertion (RI^{715}) were crossed *en masse* to *w* ; *Dr P* [*ry* + Δ2-3]99B/TM6B females. F1 *Dr* female progeny were crossed *en masse* to TM3/TM6B males. Finally, individual F2 Dr^{+} female progeny were crossed to TM3/TM6B males to produce lines

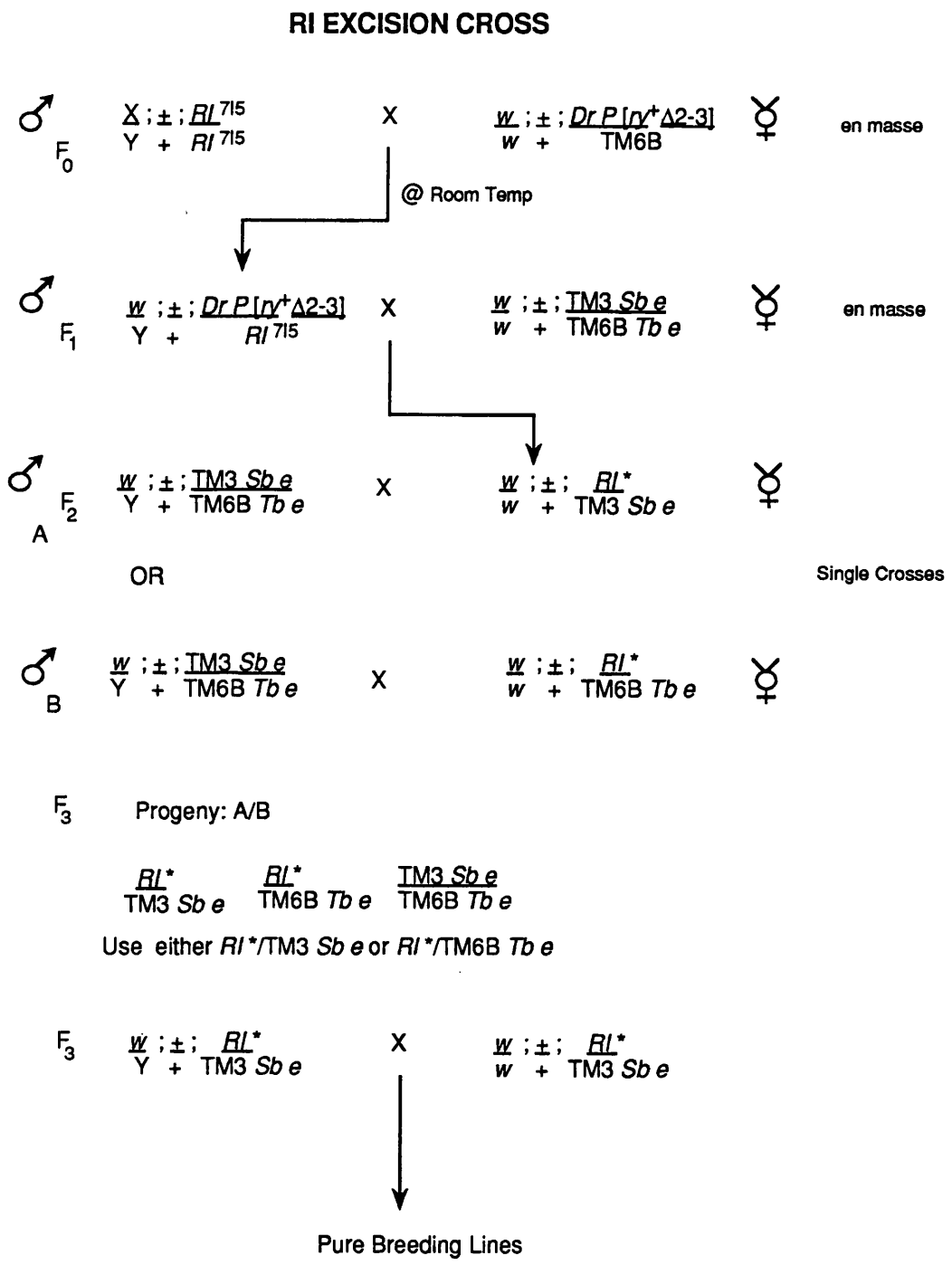


Figure 4.13. Generation of excision strains via P-element transposition. Excision strains were generated by mobilising the P-element out of the third chromosome with Δ2-3 (see table 2.1 for a description of strains used and section 4.5).

that can be tested for the presence or absence of the P-element. Approximately 1000 lines (500 F1 male and 500 F1 female derived) were generated by this scheme. Lack of a visible phenotype associated with the P-element insertion necessitated a PCR approach to try and detect excision events. The logic of this approach was as follows; DNA prepared from heterozygous revertants derived from hybrid dysgenesis was amplified with gene-specific primers flanking the insertion site (77F3 and 77Fex1) in combination with a P-element specific primer (P_{31}) and compared to wild-type and RI^{715} heterozygous controls (Figure 4.14). PCR products were separated on agarose gels; the wild-type control theoretically produced a single reaction product, the RI^{715} heterozygous control produced three reaction products (one wild-type product and two P-element dependent reaction products). The absence or change in size of a P-element dependent product(s) would be indicative of an excisional event (Figure 4.14).

To examine the possibility of detecting deletions by the above criteria, an experiment was first done to test the suitability of appropriate primer combinations. Figure 4.14, illustrates the position of the P-element insertion present in both RI mutants and the primers selected for these experiments. DNA isolated from RI^{715} heterozygous and homozygous (both female and male) mutants was amplified with various combinations of the P-element specific, P_{31} and two gene-specific primers, 77F3 and 77Fex1. Figure 4.15 (lanes 1-6), shows the results obtained. Amplification of DNA from heterozygote, homozygote RI^{715} male and female individuals with 77F3/P_{31} and 77Fex1/P_{31} primer combinations produces PCR products of anticipated size, ~271 bp and ~408 bp respectively. Similarly, amplification of DNA from female RI^{715} homozygotes with all three primers produces two P-element dependent products (lane 8). However, amplification of DNA from female RI^{715} heterozygotes with all three primers produced two P-element dependent products and two wild-type products, one of which was the anticipated size, ~610 bp (lane 7) and another amplified product that is marginally larger. I am ignorant of its origin, but its production seems to be dependent on primer complexity since amplification with 77F3 and 77Fex1 produces a single wild-type band of ~610 bp (data not shown). Lastly, amplification of DNA from male RI^{715}

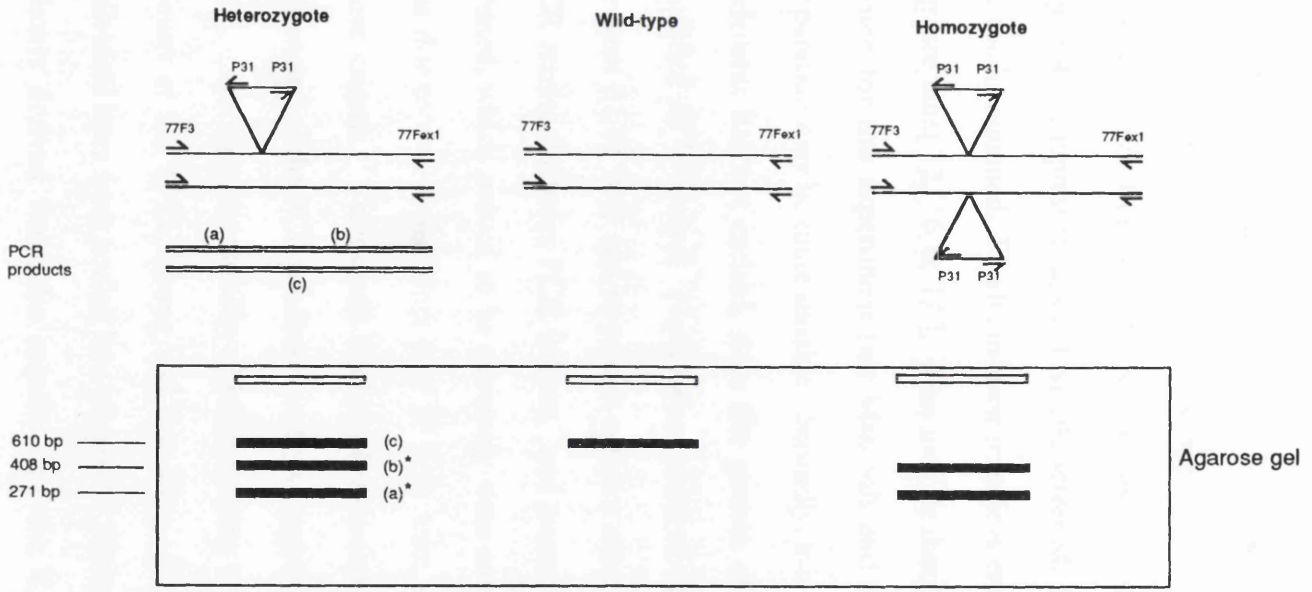
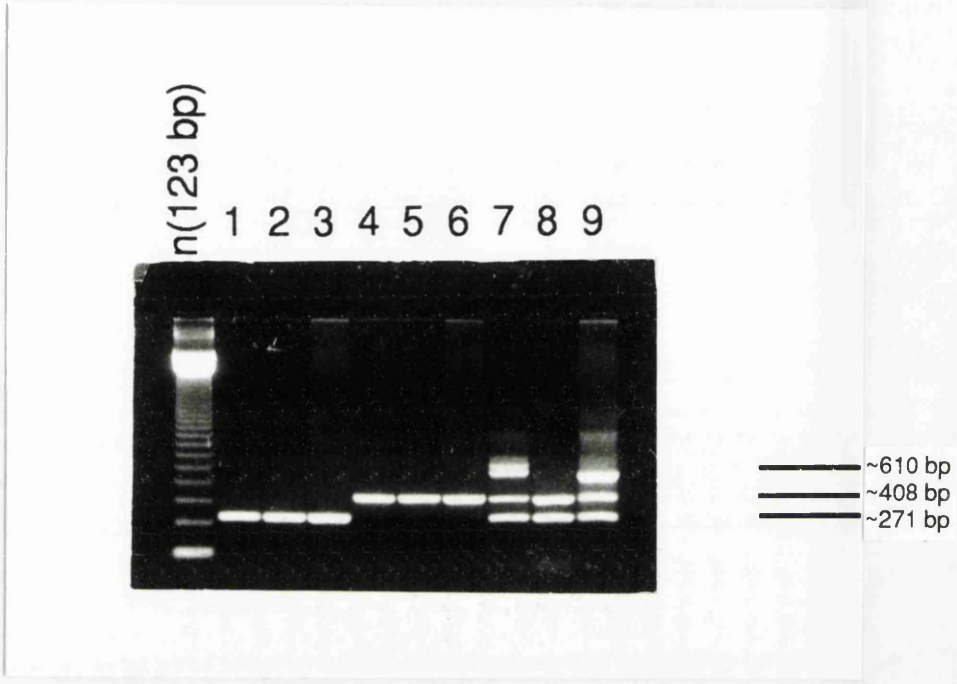


Figure 4.14. A cartoon outlining the rationale for PCR screening for deletions. Shown above is a diagram of the P-element insertion in the RI gene with potential amplification products shown as open bars (a), (b) and (c). The box below represents an agarose gel with amplification products (solid black bars) expected from a heterozygote, wild-type and homozygote. The absence or change in size of a P-element dependent product would be indicative of an excisional event.

homozygotes with all three primers produced two P-element dependent products and one wild-type product (lane 9). This wild-type product (< 610 bp) was not predicted (cf. lane 8) by the above criteria, and I believe it may represent part of the RI sequences found on the Y chromosome. Of the 1000 lines generated 500 were screened by the above strategy. As mentioned above, individual F2 Dr^{+} females were crossed to TM3/TM6B males to establish single lines. After a few days, the female was removed, DNA extracted, amplified with the appropriate primers and analysed on agarose gels (with assistance from H. Mistry). Given the repetitive nature of these experiments, Figure 4.16, representing the first 100 screened, can be considered to be an example of the results obtained. Firstly, in some lanes it is evident that there has been a PCR failure (e.g. see lanes 12, 16 & 17). This may be due in part to the rapid DNA preparation chosen for this experiment (see Materials and Methods), and a more reliable DNA preparation may be more suitable. Secondly, it is clear that for the majority of cases the P-element has not excised, since the pattern of amplified products is similar to the amplified RI^{715}control. Nonetheless, some of the results are suggestive of precise (e.g. see lanes 31 & 100) and imprecise excision events (e.g. see lanes 7 & 43). Ambiguous PCR results (includes PCR failures, and potential precise and imprecise events) were retested, which proved to be extremely time consuming. This means unfortunately, that due to time constraints only 20 lines were isolated from the first 500 flies by the above criteria. They await further characterisation by genomic Southerns or direct sequencing of the PCR products (work in progress, D. Bates and J. Hutchinson).

The second possibility of local jumping was tested by "site-selected" mutagenesis (Tower et al., 1993; Zhang and Spradling, 1993). Batches of DNA isolated from individual lines were pooled into groups of 100 and tested with a range of gene-specific primers derived from the sequence of the RI gene (see Table 2.2). No local transpositions were detected. Although Zhang and Spradling (1993) predict local transposition frequencies of 1-2%, such events are statistically less likely to occur in this experiment. Since only 1000 flies were screened and the transpositon was over a small distance (~16 kb).



Lane 1: female *RI* ^{7I5} Heterozygote amplified with 77F3/P_{31}
Lane 2: female *RI* ^{7I5} Homozygote amplified with 77F3/P_{31}
Lane 3: male *RI* ^{7I5} Homozygote amplified with 77F3/P_{31}
Lane 4: female *RI* ^{7I5} Heterozygote amplified with 77Fex1/P_{31}
Lane 5: female *RI* ^{7I5} Homozygote amplified with 77Fex1/P_{31}
Lane 6: male *RI* ^{7I5} Homozygote amplified with 77Fex1/P_{31}
Lane 7: female *RI* ^{7I5} Heterozygote amplified with 77F3/77Fex1/P_{31}
Lane 8: female *RI* ^{7I5} Homozygote amplified with 77F3/77Fex1/P_{31}
Lane 9: male *RI* ^{7I5} Homozygote amplified with 77F3/77Fex1/P_{31}

Figure 4.15. DNA isolated from *RI* ^{7I5} heterozygous (female) and homozygous (male and female) mutants was amplified with various combinations of the two gene-specific primers, 77F3 and 77Fex1 and the P-element specific primer P_{31}. n(123bp) corresponds to 123 bp DNA size marker (BRL). Amplification products were separated on a 1.5% agarose gel stained with ethidium bromide.

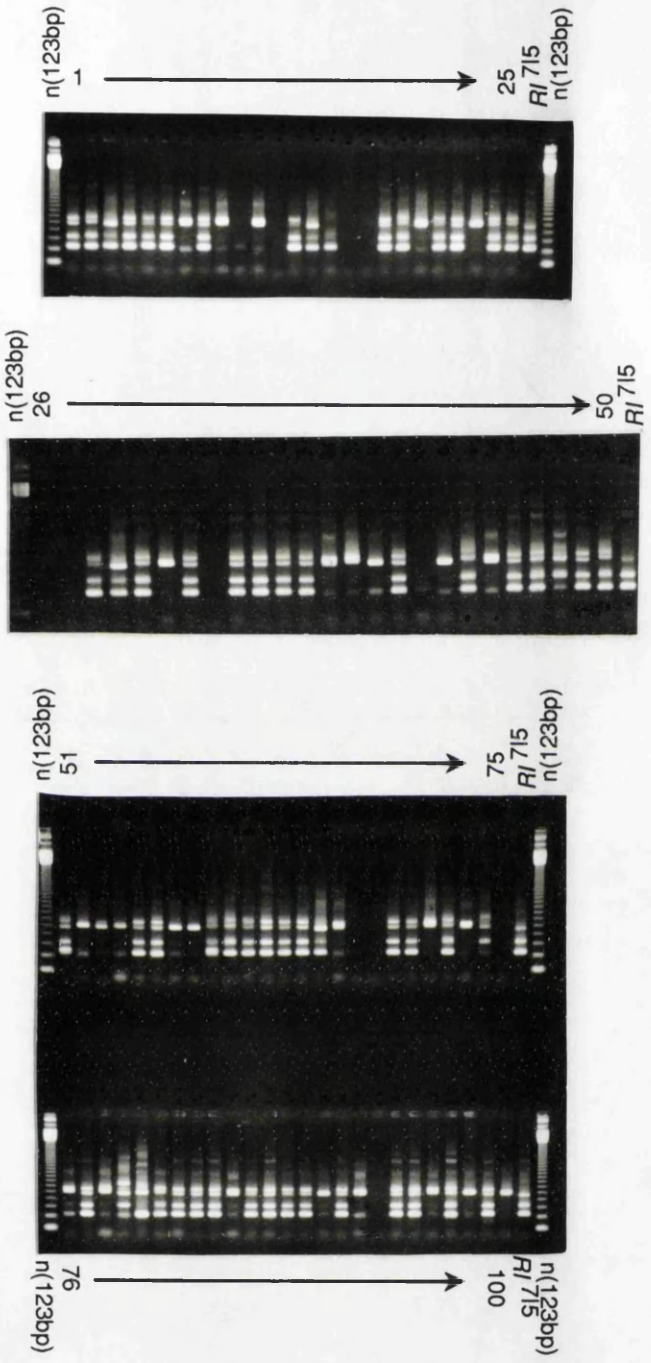


Figure 4.16. Amplification of the DNA isolated from individual F2 females (first 100 screened) using P_{31}, 77F3 and 77Fex1. Penultimate lanes in each gel show amplification of *RI* ^{715} control DNA. n(123bp) corresponds to 123 bp DNA size marker (BRL). Amplification products were separated on 1.5% agarose gels stained with ethidium bromide.

4.6 Discussion

Utilising the "site-selected" transposon mutagenesis strategy developed in chapter 3, the regulatory (RI) subunit of a cAMP-dependent protein kinase (PKA) was mutated with a view to testing the role of PKA in learning and memory in *Drosophila.* This strategy offers two major advantages. Firstly, there is no requirement for *a priori* hypothesis about an expected phenotype and secondly, mutagenised flies can be screened *en masse.* Of 12,000 flies tested, two P-element insertions were recovered at the RI locus. Both insertions occurred in the same position were of identical size and I consider them to be functionally equivalent. The insertion site was in the middle of an ~26 bp heterogeneous transcription initiation site for transcripts encoding the full length RI gene product, which is in accord with the observation that P-elements may insert preferentially into the regulatory regions of a gene (Engels, 1989).

The defective P-element insertion present in both mutant lines disrupts expression of the longest RI transcript, which encodes for the putative full length protein. It is possible that a proportion of wild-type class I mRNA is initiated 3' to the P-element insertion site and the mutant class I mRNA observed is initiated upstream from the insertion site. One would predict from eukaryotic translational models that many factors may influence the efficiency of mRNA translation. Additional P-element sequences in the mutant transcript introduce additional short reading frames upstream of the presumed RI translation start site, or secondary structure in the 5' leader region of the mutant transcript may interfere with cap accessibility and may impede the scanning of a ribosome preinitiation complex (Kozak, 1992 ; Hershey, 1991). The phenotypic consequences of this disruption to the longest RI transcript will be discussed in the next chapter.

The *in situ* hybridisation results are pleasantly in accord with the results from other genes involved in the cAMP signal transduction pathway eg. *dnc* and *rut* and *DCQ* although only at a preliminary stage, I hope to extend this analysis to the *RI* mutants, possibly using transcript-specific and P-element probes. It will also be of considerable

interest to examine the spatial and temporal distribution of RI gene product in wild-type and *RI* mutants by immunohistochemical methods in order to determine the functional loci of the gene. To this end, antibodies to two synthetic peptides are being raised at present (Y. Yung, pers. comm.).

It is not known if the RI gene is essential, but a more severe disruption may be lethal, "tagging" of this gene will facilitate the generation of deletions by imprecise excision. Since lack of a visible phenotype associated with the P-element insertion required a PCR approach to detect excision events, it remains to be seen if this strategy will allow the isolation of a variety of deletions by imprecise excision, including nulls. Alternatively, given the ever increasing numbers of fly strains in stock centres and laboratories throughout the world, each carrying a single marked P-element at a known chromosomal location, it may be possible to local jump such an element into the RI gene (see section 1.8.5), alleviating the need for a molecular approach to generate revertants. This approach has already been used successfully to target specific genes (Hamilton et al., 1993; Littleton et al., 1993). With a view to testing this approach I have recently obtained a P *lacZ* line, at polytene division 77F, from Dr V. Rodrigues. Thus, by creating a series of chromosomal deletions that remove increasingly larger portions of the RI gene from its 5' end, it may be possible to determine if individual transcription units serve different biological functions (Qiu and Davis, 1993).

The next chapter will describe the behavioural consequences of disrupting the RI gene, *in vivo*, in associative and nonassociative olfactory learning paradigms.

Chapter 5

Behavioural consequences of mutating the RI gene, *in vivo*, in associative and nonassociative olfactory learning paradigms.

5.1 Introduction

Present theories of learning and memory propose that changes in the flow of information in neuronal networks executing specific behaviours underlie the ability of the nervous system to modify these behaviours (Hawkins et al., 1993). Studies of the alterations that take place in nervous systems during learning can be pursued by direct strategies such as monitoring and deciphering the activities of the nerve cells, processes and synapses that participate in the behavioural task. The sea snail, *Aplysia californica*, has become a model organism for such studies as it has relatively simple networks and identifiable large cells that can be associated with simple behaviours (Kandel, 1991).

Despite its small size, genetic dissection of *Drosophila melanogaster* offers a exceptionally powerful tool to study learning and memory. The rationale for the genetic dissection approach to memory is simple. Genes code for the macromolecules that may comprise the structural and functional building blocks of biological memory systems. By altering each of the appropriate genes separately, one can produce specific lesions in the learning apparatus, thus disrupting the ability to learn and remember. Genetic, biochemical and electrophysiological comparisons made between wild-type and mutant organisms, which differ at only one gene, may reveal the nature of the gene product and the molecular component required for normal learning.

Learning is the process of acquiring information about the environment. The memory process is the retention of acquired information and the ability to retrieve it for later use. Evidence drawn from experiments utilising specific "blockers" of protein synthesis in vertebrate and invertebrate learning studies suggest that memory can be divided into at least two phases: the short-term memory phase, which does not require new protein synthesis, and the long-term memory phase which requires new protein synthesis (Kandel, 1991). In addition, there would appear to be no defined temporal marker to separate short-term and long-term memory (Montarolo et al., 1986). It is clear from studies with *Aplysia* that there is a narrow time window for the acquisition of long-term memory, it being most sensitive to disruptions during and immediately after

training (Goelet et al., 1986). In *Drosophila*, this time window extends to 30-60 minutes after associative training with negative reinforcement, as shown by Quinn and Dudai (1976) and Tempel et al. (1983) who anaesthetised the flies briefly at 4°C at various times after training.

There are several different classes of learning. Associative learning concerns the relationship between two stimuli or a stimulus and the animal's own behaviour. Classical conditioning is a form of associative learning in which the animal learns that the presentation of one type of stimulus predicts the occurrence of a second (Mackintosh, 1983; Rescorla, 1988; Kupfermann, 1991). Nonassociative learning concerns the presentation of a single type of stimulus. Specifically, habituation is a nonassociative form of learning represented as a decrease in a reflex response due to the repetitive presentation of a stimulus (Thompson and Spencer, 1966; Kupfermann, 1991), whereas sensitisation is a form of nonassociative learning in which a strong stimulus enhances subsequent reflex responses of the animal (Kupfermann, 1991).

Drosophila has well established classical and molecular genetics and a variety of strategies exist for producing mutations and cloning genes of interest (Rubin, 1988). Equally important is its ability to display different types of learning and memory, both associative and nonassociative (Aceves-Pina, et al., 1983; Corfas and Dudai, 1989; Dudai, 1988; Duerr and Quinn, 1982 ; Tully and Quinn, 1985; Tully, et al., 1990) in appropriate experimental paradigms, facilitating the isolation of mutations affecting these processes (Quinn, et al., 1974; Dudai et al., 1976; Tully and Quinn, 1985; Boynton and Tully, 1992). Behavioural conditioning is not unique to adult flies; larvae can also learn to associate olfactory cues and negative reinforcement (Aceves-Pina and Quinn, 1979) and the memory of odour habituation is preserved through metamorphosis (Manning, 1967). Although flies elicit a wide variety of behaviours amenable to study, most studies, including the results presented in this chapter, use a olfactory classical conditioning paradigm (Tully and Quinn, 1985).

In the olfactory classical conditioning paradigm, 100-150 flies are enclosed in a chamber with an electrifiable copper surface (Figure 5.1) and are trained by exposing

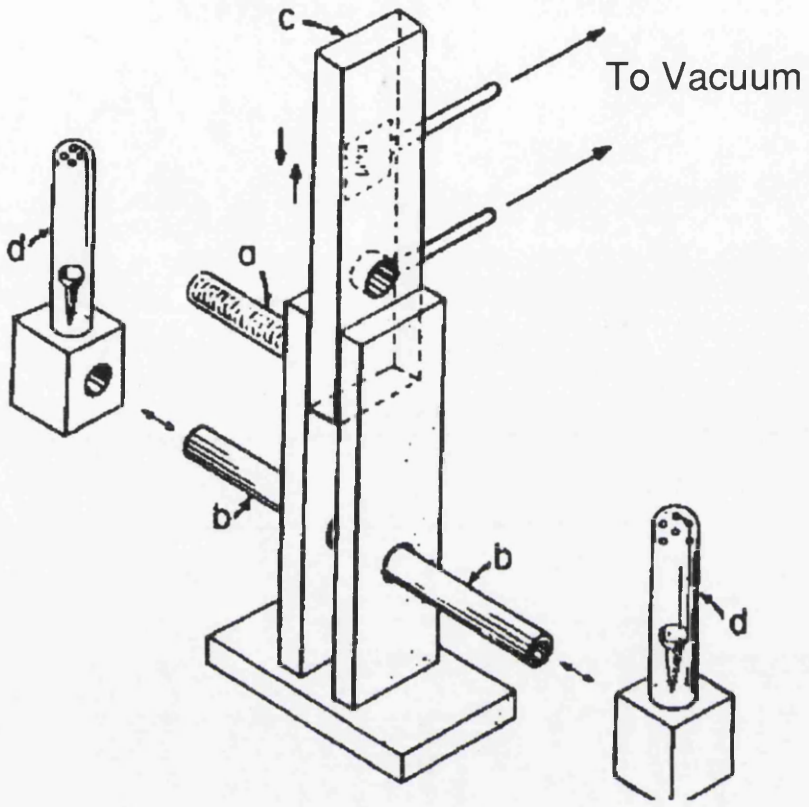


Figure 5.1. Classical conditioning apparatus. About 150 flies are sequestered in a closed grid tube (a) and trained by exposing them sequentially to two odours (3-octanol or 4-methylcyclohexanol), which are housed in odour tubes (d) and delivered in air currents. Flies receive twelve 60V (DC) shock pulses during a 60 sec presentation of the first odour (CS^+) but not during a 60 sec presentation of the second, control odour (CS^-). To test for conditioned odour avoidance, flies are transported via a sliding centre compartment (c) to a T-maze choice point between converging currents of the two odours. During this 120 sec test trial, most (wild-type) flies avoid the T-maze arm (b) containing the CS^+ by walking into opposite arm containing CS^-. After Tully and Quinn, 1985.

them sequentially to two odours delivered in air currents. Flies receive twelve 1.25 sec 60 V (DC) electric shock pulses every 5 sec (unconditioned stimulus, US) during a 60 sec presentation of the first odour (conditioned stimulus, CS^+) but not during a 60 sec presentation of the second odour (control stimulus, CS^-). To test for conditioned avoidance, flies are tapped gently into a sliding compartment and transported to the T-maze choice point, between converging currents of the two odours (Figure 5.1). Typically, 90% of wild-type flies avoid the shock-associated odour after training, less than 5% avoid the CS^-, and less than 5% remain in the middle of the T-maze choice point, resulting in a learning index of approximately 90% (Tully and Quinn, 1985). Memory curves for wild-type (Canton-S) and three mutants over a 24 hour period are illustrated in Figure 5.2. Tully and Quinn, (1985) demonstrated empirically that the average learning index for a population of flies is not biased by nonassociative changes in avoidance behaviour: conditioned avoidance is not observed if flies are presented only with shock or only odours, or if the two stimuli are presented separately (Figure 5.3). Thus the avoidance behaviour is induced by pairing temporally the odour with shock. This classical conditioning procedure produces very strong conditioned avoidance responses in wild-type flies and moderate levels of learning in mutants. More importantly, in this paradigm, associative learning levels observed in wild-type flies are significantly higher than those of mutant flies, clearly demonstrating differences in memory retention after training.

In order to study the behavioural consequences of mutating the RI gene in an associative olfactory learning paradigm (Tully and Quinn, 1985), we initiated a collaboration with Dr. T. Tully (Cold Spring Harbor). Dr. Tully has considerable experience with this paradigm and the following experiments were initiated in his laboratory during a four week visit. Although I was able to carry out pilot experiments with the olfactory learning apparatus, time constraints imposed by quarantine restrictions and subsequent amplification of fly strains meant that the final data was collected by Maria del Vecchio, an experienced technician in the Tully laboratory, after my visit

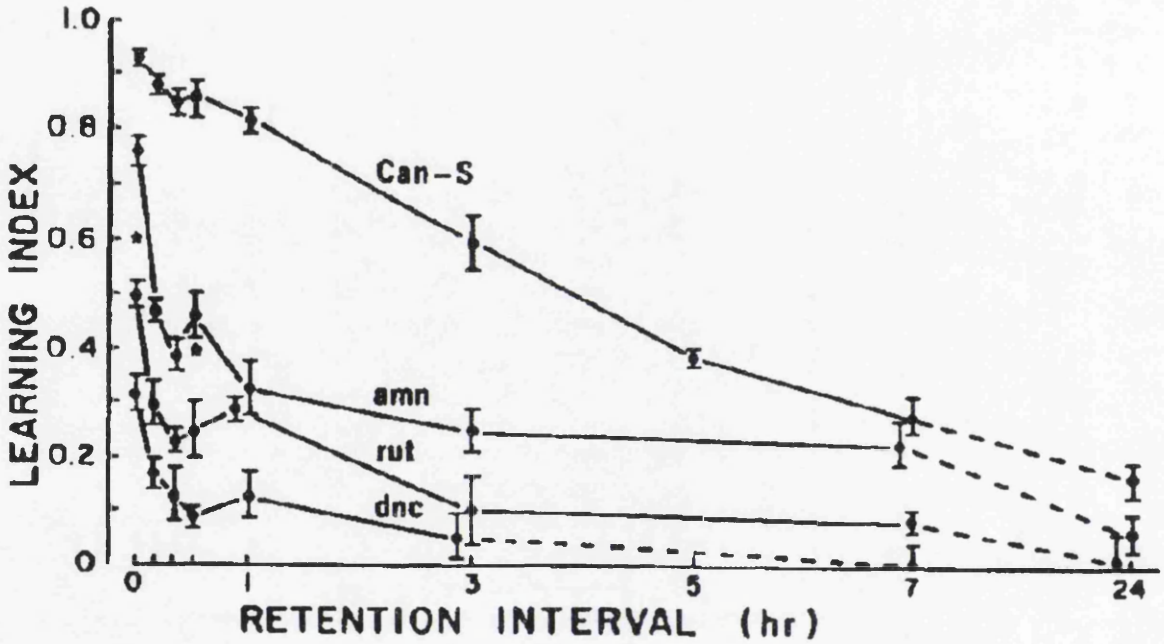


Figure 5.2. **Memory retention in normal and mutant flies.** Different groups of flies received 12 shock pulses during training and then were tested at various intervals afterwards. Retention curves are drawn for wild-type (Can-S), *amnesiac* (amn), *rutabaga* (rut), *dunce* (dnc) flies. Each point represents 4 experiments. After Tully and Quinn, 1985.

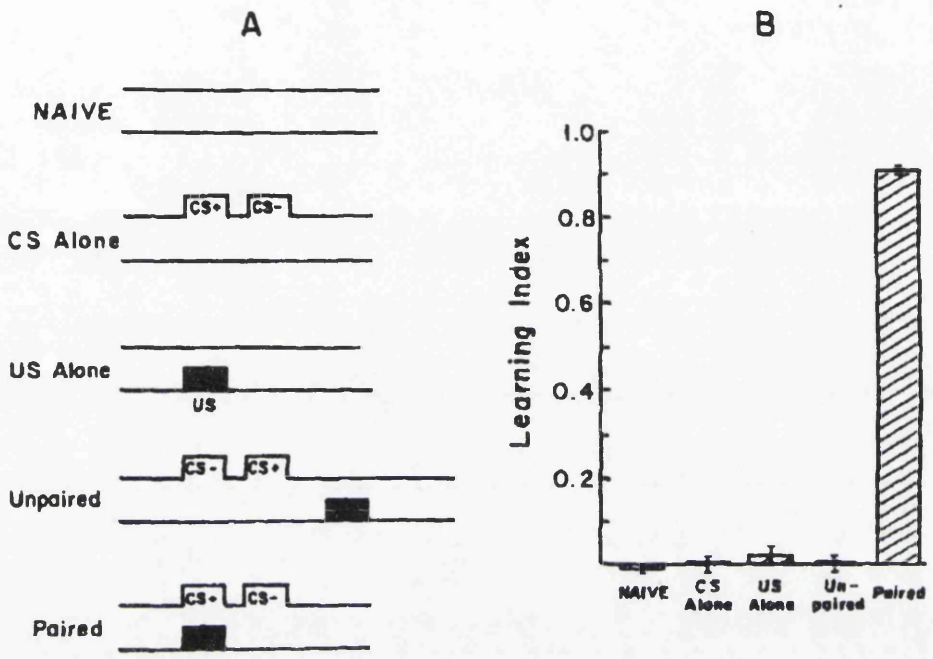


Figure 5.3. Nonassociative control procedures for classical conditioning.

A: During training, different groups of flies received no stimuli (Naive), odours alone (CS Alone), electric shock alone (US Alone), odours and shock separated in time (Unpaired), or shock in the presence of (one) odour (Paired). B: Each of the nonassociative control procedures yield a learning index of zero. In contrast, temporal pairing of odour and shock produces a significant learning index. Thus, the learning index is a measure of associative learning unbiased by nonassociative effects, which actually are produced. After Tully and Quinn, 1985.

concluded. In addition, I was able to carry out preliminary experiments in a nonassociative learning paradigm, habituation of the jump reflex to olfactory cues.

5.2 Perturbation of the RI gene disrupts olfactory associative learning

Genetic background modifiers are known to affect both associative learning specifically, and other peripheral behaviours, such as olfactory acuity and shock reactivity, required for classical conditioning (Gailey, et al., 1991; Boynton and Tully, 1992; Dura et al., 1993). These observations necessitated the isogenisation of the RI^{7I5} and RI^{11D4} strains with a high learning index, wild-type Canton-S (*C-S*). To do this, the RI^{7I5} and RI^{11D4} strains were outcrossed for more than six generations with the *C-S* strain. The P-element insertions were followed as amplification products, allowing free recombination to occur. After at least six generations both RI^{7I5} and RI^{11D4} were "Cantonised" for both the autosomes and X chromosome and only the P-element at the RI locus remained (see Chapter 4, section 4.2.4).

To test the ability of the mutant lines to learn and/or remember, a classical conditioning paradigm was employed (Tully and Quinn, 1985). For a description of the training and testing procedures, see section 2.1.3. This type of associative learning paradigm tests the ability of the fly to learn that the presentation of one stimulus predicts the occurrence of a second. The flies are exposed to two different odours sequentially, but only one odour is coupled temporally with an electric shock. Wild-type flies can learn and remember the association between odour and shock as judged by their subsequent avoidance of the odour coupled with electric shock when the flies are given a choice between the two odours. Both homozygous *RI* mutant strains and a wild-type Canton-S (*C-S*) strain were trained by alternate exposure to the odours 3-octanol (OCT) and 4-methylcyclohexanol, (MCH), one of which is paired with an electric shock (CS^+) and a second, control odour without reinforcement (CS^-).

All the lines were then tested at intervals for their retention of the association. In addition, all the lines were tested for defects in the sensory modalities involved in the classical conditioning paradigm, namely olfactory acuity and shock reactivity.

5.2.1 Memory Retention

RI ^{7I5}, *RI* ^{11D4} and *C-S* control flies were tested at specific retention intervals, (15, 30, 60 and 180 minutes) after classical conditioning. In practice it is impossible to measure a retention interval of 0 min. So for retention intervals of 0 min, flies were actually transferred to the T-maze 1.5 min after training. After test trials, an equal number of performance indices (PI) was then calculated for each genotype at each retention interval, using the formulae described in section 2.1.3. Table 5.1 summarises the data obtained for the three genotypes over the first 3 hr after training.

Genotype	time (min)	*n*	PI (mean)	Std (PI)	SEM (PI)
C-S	0	6	87.8	1.9	0.8
C-S	15	6	70.5	3.3	1.3
C-S	30	6	67.4	5.2	2.1
C-S	60	6	63.7	9.7	4.0
C-S	180	6	42.3	6.2	2.5
RI ^{7I5}	0	6	67.9	7.8	3.2
RI ^{7I5}	15	6	51.9	6.7	2.7
RI ^{7I5}	30	6	45.5	5.9	2.4
RI ^{7I5}	60	6	35.8	11.8	4.8
RI ^{7I5}	180	6	27.6	12.8	5.2
RI ^{11D4}	0	6	66.1	4.9	2.0
RI ^{11D4}	15	6	57.5	8.5	3.5
RI ^{11D4}	30	6	48.8	6.2	2.5
RI ^{11D4}	60	6	37.2	3.9	1.6
RI ^{11D4}	180	6	31.1	8.3	3.4

Table 5.1. Mean PI scores for the three genotypes (*C-S*, *RI* ^{7I5}, *RI* ^{11D4}) over the first 3 hr after training standard deviations (Std) and standard errors (SE) for the data presented in Figure 5.4.

Figure 5.4, shows memory retention over the first 3 hr after training for *RI* ^{7I5} and *RI* ^{11D4} and *C-S* control flies. Clearly, mean PIs are lower in mutant *RI* ^{7I5} and *RI* ^{11D4} flies at all retention intervals. A two way analysis of variance (ANOVA) (Table 5.2) reveals a significant effect of both GENOTYPE ($F_{[2, 75]} = 68.5$, $P < 0.001$), and of TIME ($F_{[4, 75]} = 74.0$, $P < 0.001$), but no significant interaction between GENOTYPE X TIME ($F_{[8, 75]} = 1.3$, $P > 0.05$).

source	df	SS	MS	F	*P*
time	4	16481.0	4120.3	74.0	< 0.001
genotype	2	7634.0	3817.0	68.5	< 0.001
interaction	8	566.5	70.8	1.3	> 0.05
error	75	4178.0	55.7		
total	89	28859.5			

Table 5.2. Analysis of variance for effects of genotype and time on PIs.

These results indicate that the initial difference in conditioned avoidance between *RI* ^{7I5}, *RI* ^{11D4} and *C-S* control flies remained constant during the first 3 hr of memory decay. In other words, the kinetics of memory decay are roughly that of the wild-type control. When averaged over all retention intervals, *RI* ^{7I5} and *RI* ^{11D4} scores were approximately 75% of those of *C-S* controls.

5.2.2 Olfactory Acuity

To test whether poor performance of *RI* ^{7I5} and *RI* ^{11D4} flies in conditioning experiments was a secondary result of a diminished capacity to smell the odour cues, naive *RI* ^{7I5}, *RI* ^{11D4} and *C-S* control flies were given a choice between either OCT or MCH versus air in the T-maze. Tables 5.3 and 5.4 summarise the data obtained from this experiment:

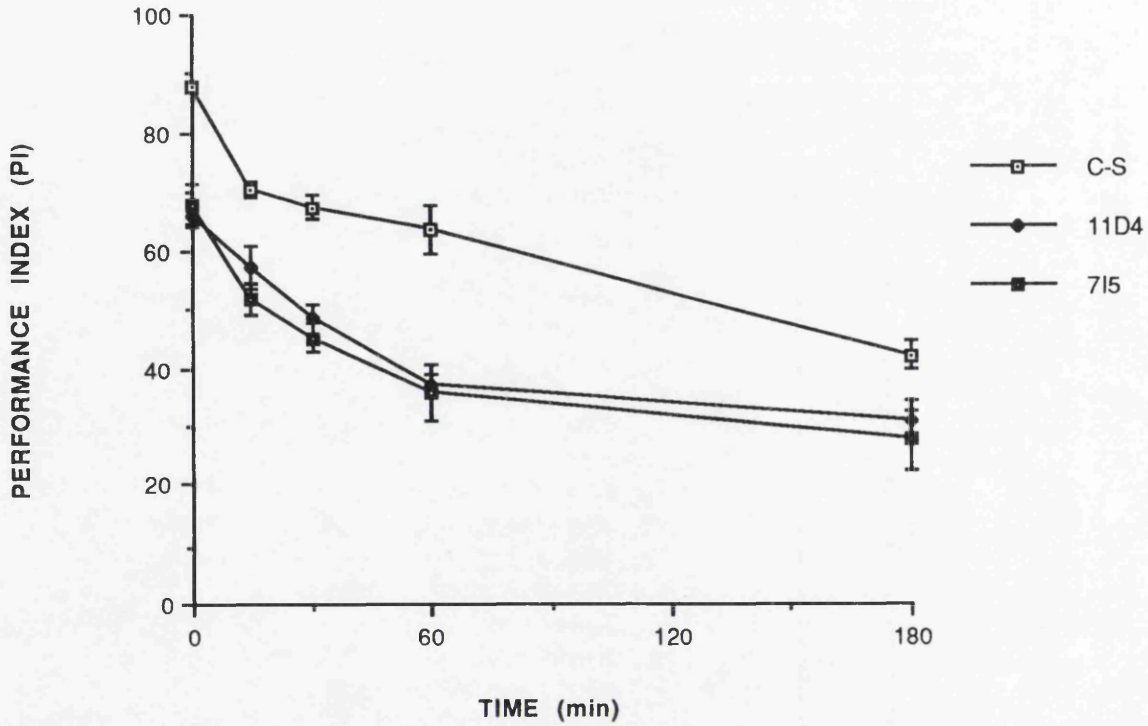


Figure 5.4. **Memory retention in mutant and control flies.** Flies were trained by sequential exposure to the odours OCT and MCH, one of which was paired with an electric shock. They were then tested at intervals for their retention of the association. The performance index (PI) is a function of the percentage of flies that avoid the shock associated odour. Both lines exhibit a consistent reduction in learning and memory at all retention intervals. n = 8 PIs for each genotype at each retention interval. Raw data is presented in the appendix. Error bar, SEM.

Genotype	*n*	PI (mean)	Std (PI)	SEM (PI)
C-S	16	69.1	7.7	1.9
RI^{7I5}	16	61.3	14.3	3.6
RI^{11D4}	16	66.8	9.7	2.4

Table 5.3. Mean PI scores for OCT *vs.* air, standard deviations and standard errors for the data presented in Figure 5.5a.

Genotype	*n*	PI (mean)	Std (PI)	SEM (PI)
C-S	16	70.9	11.8	2.9
RI^{7I5}	16	65.5	11.8	2.9
RI^{11D4}	16	68.4	12.3	3.1

Table 5.4. Mean PI scores for MCH *vs.* air, standard deviations and standard errors for the data presented in Figure 5.5b.

Figure 5.5 shows mean PIs for avoidance of OCT *vs.* air and MCH *vs.* air for each of the strains. A one way ANOVA reveals there is no significant difference between GENOTYPE ($F_{[2, 45]} = 2.17$, $P = 0.126$), for OCT vs. air (Table 5.5).

source	df	SS	MS	F	*P*
genotype	2	518	259	2.17	0.126
error	45	5375	119		
total	47	5893			

Table 5.5. Analysis of variance for differences in mean performance indices with genotype, for OCT *vs.* air.

A one way ANOVA reveals there is no significant difference between GENOTYPE ($F_{[2, 45]} = 0.85$, $P = 0.436$), for MCH vs. air (Table 5.6).

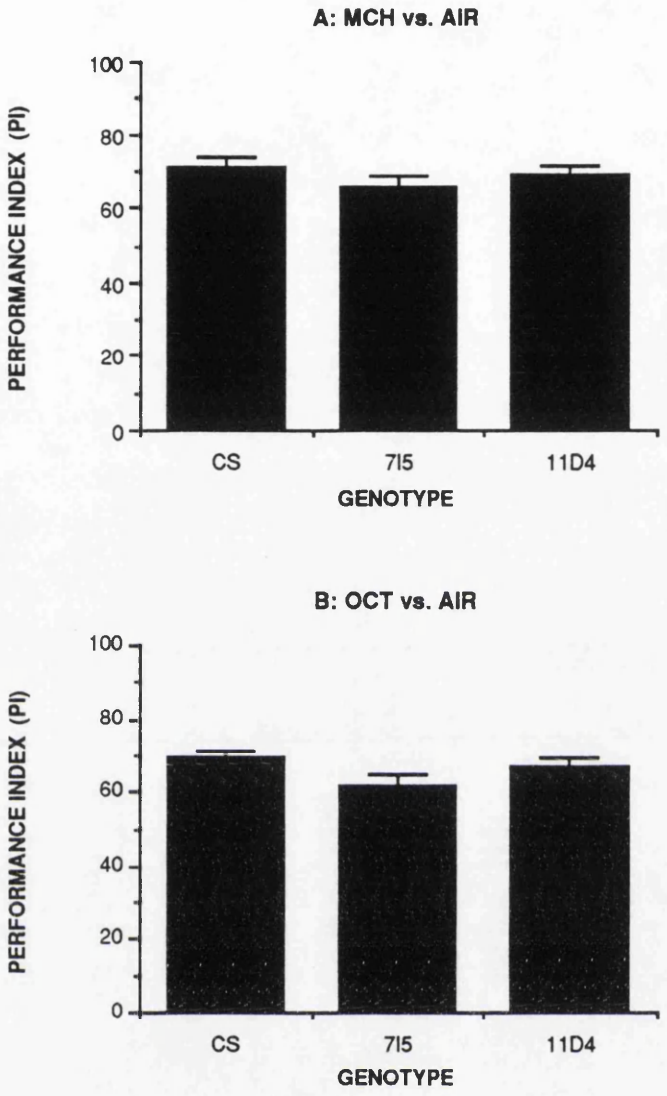


Figure 5.5. **Olfactory acuity in mutant and control flies.** Each genotype was tested for olfactory avoidance by exposing them in the T-maze to converging currents of air-one of which delivered odourant and the other "fresh" air. The percentage of flies avoiding the T-maze arm containing the odour by running into the opposite arm was used to calculate a PI. (A) Avoidance responses to 4-methylcyclohexanol (MCH). (B) Avoidance responses to 3-octanol (OCT). Olfactory acuity in mutant flies did not differ significantly from that in wild-type controls. n = 16 PIs for each genotype at each odour. Raw data is presented in the appendix. Error bar, SEM.

source	df	SS	MS	F	*P*
genotype	2	242	121	0.85	0.436
error	45	6441	143		
total	47	6683			

Table 5.6. Analysis of variance for differences in mean performance indices with genotype, for MCH *vs.* air.

Thus, no significant differences (*P*s > 0.05) for avoidance of MCH or OCT were detected among the 3 strains. These results suggest that olfactory acuity for the two odours used in classical conditioning experiments was roughly normal in *RI* ^{7I5} and *RI* ^{11D4} strains and cannot account for the low performance levels observed during conditioning experiments.

5.2.3 Shock Reactivity

Poor performance of *RI* ^{7I5} and *RI* ^{11D4} flies in conditioning also might result from an inability to perceive and escape from an electric shock. To assess this "peripheral " behaviour, all three strains were individually exposed in the T-maze to electric shock pulses, table 5.7 summarises the results from this experiment.

Genotype	*n*	PA (mean)	Std (PI)	SEM (PI)
C-S	8	84.8	6.0	2.1
RI ^{7I5}	8	82.8	5.8	2.1
RI ^{11D4}	8	82.5	5.2	1.8

Table 5.7. Mean PA scores, standard deviations and standard errors for the data presented in Figure 5.6.

Figure 5.6, shows mean percent avoidance (PA) scores from shock reactivity assays in these three strains. A one way ANOVA reveals no significant differences between the GENOTYPES ($F_{[2, 21]} = 0.39$, $P = 0.684$) in their perception of shock (Table 5.8) .

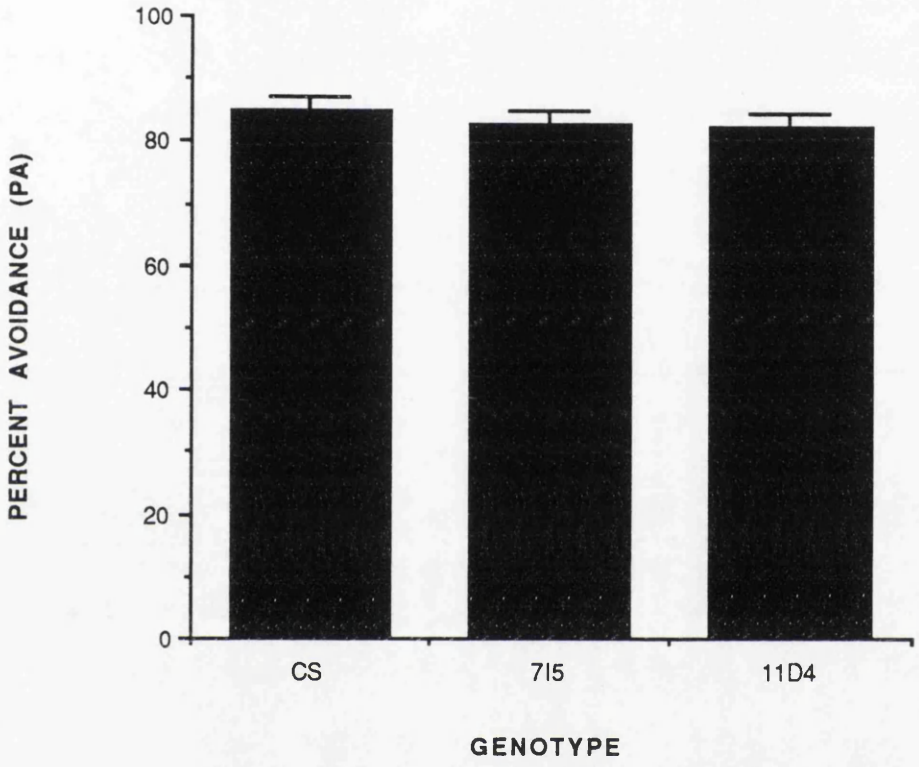


Figure 5.6. **Shock reactivity in mutant and wild-type control flies.** Each genotype was tested for shock reactivity by introducing the flies into one arm of the T-maze, delivering 1-sec pulses of electric shock to that arm for 60 sec while allowing the flies to escape to opposite (unshocked) T-maze arm. Shock reactivity was quantified as the PA of flies avoiding (escaping from) the shocked T-maze arm. Shock reactivity in mutant flies did not differ significantly from that in wild-type controls. $n = 8$. Raw data is presented in the appendix. Error bar, SEM.

PAs did not differ among *C-S*, *RI* ^{7I5} and *RI* ^{11D4} strains, indicating that low performance levels in the *RI* strains during conditioning experiments cannot be accounted for by the effects of this peripheral behaviour.

source	df	SS	MS	F	*P*
genotype	2	25.0	12.5	0.38	0.684
error	21	677.9	32.3		
total	23	702.9			

Table 5.8. Analyses of variance for differences in mean PAs with genotype.

5.3 Habituation of the Jump response to Olfactory cues

This paradigm is based on an olfactory jump assay developed by McKenna et al. (1989) (Figure 5.7). They observed that a jump response was elicited by exposing single male flies to a 3 second pulse of 10% benzaldehyde in a constant current of air. Tully (pers. comm.), who has semi-automated the paradigm, has observed that initially all flies tested will jump in response to this unpleasant stimulus, but the percentage of flies that jump in response to this odour stimulus decreases with repeated presentations of the odour.

Exploiting this habituative property, Koss and Tully (in preparation) have tested wild-type and mutant strains (*dnc*, *rut* and *amn*) in this paradigm. Individual male flies of the appropriate genotype were habituated by exposing them repeatedly to 10% benzaldehyde for 4 seconds at 1 minute intertrial intervals (ITI). They judged that each fly had habituated when it failed to jump during the 4 second odour presentation in four consecutive trials (4 "no-jumps"). A fly's habituation score (HS) was the number of trails required to obtain a block of 4 "no-jumps" (trials to criterion). More trials to criterion are required for a fly to habituate if the ITI is increased. To test dishabituation, each fly, 2 minutes after reaching criterion, was vortexed in its test tube for 90 seconds to dishabituate the jump reflex and exposed to a final 4 second odour presentation. The percentage of flies that failed to jump during this test trail reflected the average amount

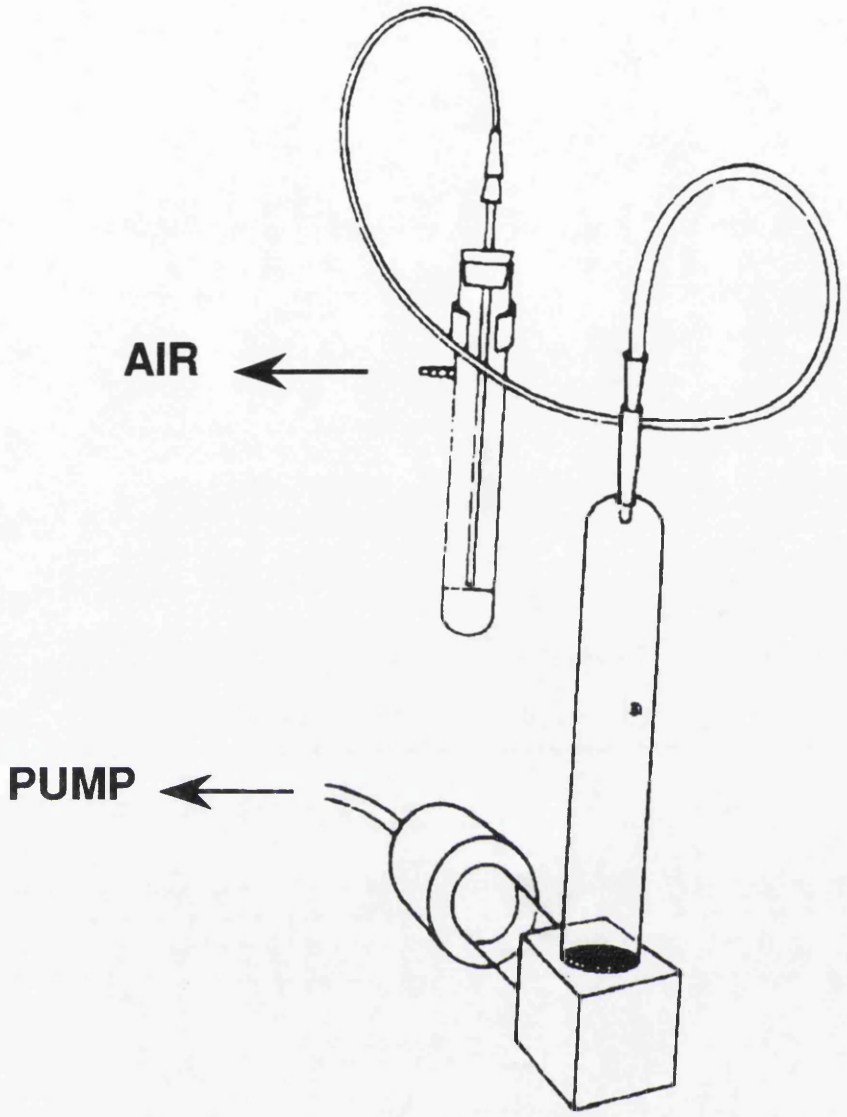


Figure 5.7. Apparatus for testing jump response to olfactory cues. A single male is introduced into the inverted plastic test tube on a lucite base. Air (through distilled water) is drawn through the chamber by a vacuum source (1000ml/min). At predetermined time intervals, a computer controlled, 3-way solenoid valve switches from "fresh" air to one that is bubbled through distilled water and then through 10% benzaldehyde in heavy mineral oil. (Figure taken from McKenna et al., 1989)

of dishabituation for each genotype tested. Memory retention was tested at varying time intervals after reaching criterion by exposing each fly to a final 4 second odour presentation. The percentage of flies that jumped during this test trial reflected the average amount of spontaneous recovery (a measure of memory retention) for each genotype tested.

Koss and Tully (in preparation) observed a number of trends when testing the learning and memory mutants. Firstly, *rutabaga* mutants take longer to habituate relative to wild-type controls, spontaneous recovery was faster than normal and dishabituation was normal. Secondly, *dunce* mutants habituate faster or slower than wild-type, depending on the length of the ITI (as ITI increases, *dnc* mutants take longer to habituate), spontaneous recovery was faster than normal and dishabituation was extremely low. Lastly, *amnesiac* mutants had normal habituation, spontaneous recovery and dishabituation.

These observations in combination with studies from other associative and nonassociative paradigms (Duerr and Quinn, 1982; Corfas and Dudai, 1989; Tully, 1990) suggest that mutations in cAMP metabolism may affect processes that are common to both forms of learning.

To test the possibility that mutations in the RI gene affect processes that are common to both associative and nonassociative learning, isogenised homozygous *RI* mutant and wild-type Canton-S (*C-S*) males were assayed in this nonassociative learning paradigm.

5.3.1 Habituation of the jump reflex to olfactory cues in wild-type and *RI* mutants

Before testing, males of the appropriate genotype were collected under CO_2 anaesthesia on the day of eclosion and stored in food vials (~10 males/vial) at 25°C for 2 days. To reduce daily variations in overall activity, locomotor activity in 2-day-old males was determined on the day of the experiment. Locomotor activity was measured using the countercurrent phototaxis apparatus of Benzer, (1967) (see section 2.1.3). In this way,

only active healthy males were chosen for these experiments. Flies were transferred to the habituation apparatus without exposure to anaesthesia. Individual wild-type (*C-S*), *RI* ^{7I5} and *RI* ^{11D4} mutant males were exposed to 4 second pulses of 10% benzaldehyde every 1 minute, until they had reached criterion i.e. 4 "no-jumps". In this way a habituation score (HS) was determined for individual flies. Table 5.9, shows mean habituation scores (HS) for each of the strains tested at a 1 minute ITI for 5 separate experiments. A two way ANOVA (Table 5.10) reveals a significant difference between EXPERIMENT ($F_{[4, 225]} = 2.83$, $P < 0.05$), and mean habituation scores, but no significant differences between GENOTYPE ($F_{[2, 225]} = 0.038$, $P > 0.05$) and mean habituation scores and no significant interaction between GENOTYPE X EXPERIMENT ($F_{[8, 225]} = 0.36$, $P > 0.05$).

Genotype	Exp 1 (HS)	Exp 2 (HS)	Exp 3 (HS)	Exp 4 (HS)	Exp 5 (HS)
C-S	6.9 (±0.9)	7.9 (±1.0)	6.3 (±1.1)	8.4 (±1.1)	5.8 (±1.0)
RI ^{7I5}	6.4 (±1.1)	7.3 (±1.0)	8.0 (±1.3)	8.0 (±0.9)	6.2 (±0.7)
RI ^{11D4}	6.1 (±1.0)	7.6 (±0.8)	8.2 (±1.1)	8.7 (±1.0)	6.0 (±0.8)

Table 5.9. Mean Habituation scores ± SE for all genotypes tested. $n = 16$

source	df	SS	MS	F	*P*
experiment	4	181.8	45.3	2.83	< 0.05
genotype	2	1.2	0.6	0.038	> 0.05
interaction	8	45.8	5.7	0.36	> 0.05
error	225	3598.5	16.0		
total	239	3826.6			

Table 5.10. Analysis of variance for effects of genotype and experiment on HSs.

It is clear that there is experiment to experiment variation, but within any one experiment the mean habituation scores are not significantly different. Why this should

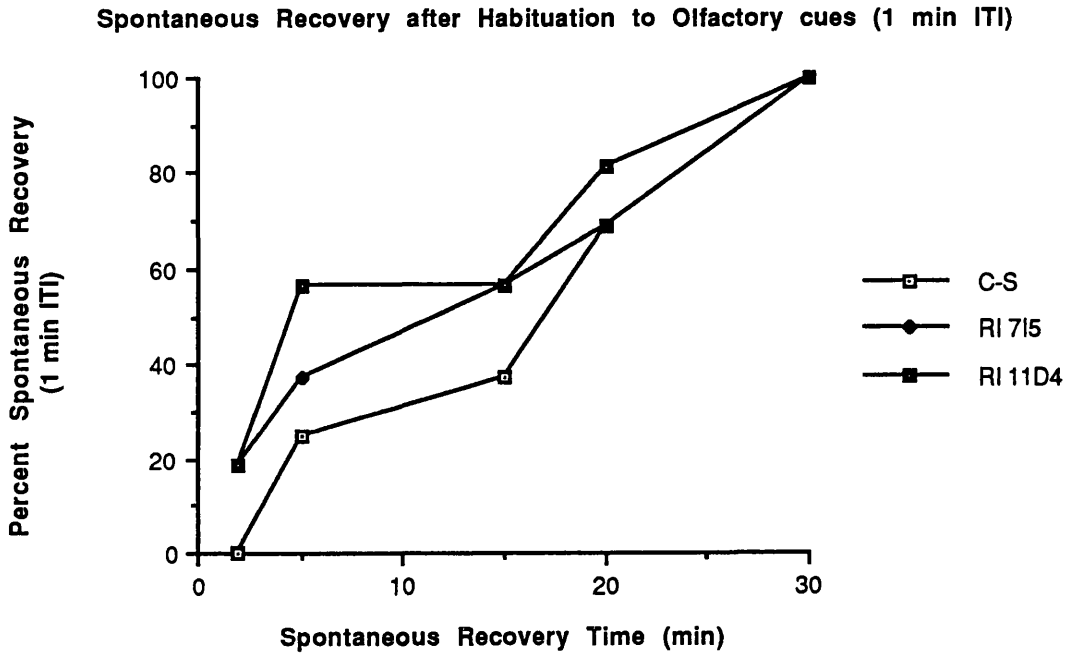


Figure 5.8. Spontaneous Recovery in wild-type (*C-S*), RI^{7I5} and RI^{11D4} mutants tested (see text) for spontaneous recovery at specific time intervals, (2, 5, 15, 20 and 30 minutes) after reaching criterion. n=16. Raw data is presented in the appendix.

be is unclear, but it may reflect unknown variables associated with this paradigm.

Tully's data (pers. comm.) suggest that after habituating, 80% of wild-type flies will jump in response to the odour stimulus if they have been dishabituated by a strong stimulus i.e. vortexing for 90 seconds in their test tube. Although I was able to demonstrate habituation to the odour stimulus, I was unable to reliably demonstrate dishabituation. Nonetheless, I decided to exploit the observation that flies exhibit a time-dependent spontaneous recovery after habituating.

Individual wild-type (*C-S*), RI^{7I5} and RI^{11D4} mutants were tested (as above) for spontaneous recovery at specific time intervals, (2, 5, 15, 20 and 30 minutes) after reaching criterion. After testing, the percentage of flies spontaneously recovering was calculated for each genotype and at each time interval (Figure 5.8). After a 2 min spontaneous recovery time, the *RI* mutants recovery is higher than that of *C-S*. This difference remains for at least the first 15 min. After 20 min the *RI* mutants are spontaneously recovering at a rate similar to *C-S*. Although only a trend, it would appear that the RI mutants spontaneously recover at a faster rate than *C-S*. This may reflect a defect with memory retention.

5.4 Discussion

Using the "site-selected" transposon mutagenesis strategy described in the previous chapters, the regulatory (RI) subunit of a cAMP-dependent protein kinase gene (PKA), was mutated with a view to testing the role of PKA in learning and memory in *Drosophila*. Two lines were recovered at the RI locus, from a P-element insertional mutagenesis, both containing a defective P-element insertion within a ~36 bp region containing multiple transcription start sites which give rise to transcripts encoding the full-length RI gene product. Northern analysis indicates that a transcript of a discrete novel size accumulates in both mutant lines. Both lines are homozygous viable.

The isogenised homozygous *RI* mutants were tested immediately after discriminative classical conditioning (Tully and Quinn, 1985) using odours as

conditioned stimulus and electric shock as the unconditioned stimulus. On average the two *RI* mutants show only 75% of wild-type levels of conditioned avoidance. This "learning" deficit remains constant during the first 3 hours of memory retention (Figure 5.4) thereby producing a mutant memory curve that is parallel to that of the *C-S* control flies.

Another feature of the olfactory learning of *RI* mutants is that there is no rapid initial memory decay observed with the *dunce*, *rutabaga*, and *amnesiac* mutants of *Drosophila* (Figure 5.2, Tully and Quinn, 1985; Tully, 1990). The kinetics of memory loss are comparable to those observed for other learning mutants such as *latheo* (Boynton and Tully, 1992), *linotte* (Dura et al., 1993; Préat, 13th European *Drosophila* Research Conference, 1993) and a protein phosphatase I mutant (Asztalos et al., 1993). The initial learning decrement seen with *RI* mutants suggests that learning (acquisition), rather than memory, is disrupted. Clearly, the poor performance levels for *RI* mutants do not result from an inability to perceive an odour or escape from a shock, as both "peripheral" behaviours were normal in *RI* mutants. In addition, analysis of the *RI* mutants for circadian rhythm and general locomotor activity behaviours Emery (pers. comm.), shows them to be normal for both their 24 hour rhythm and level of locomotor activity. I conclude that associative learning *per se* is defective in these mutants.

The isogenised homozygous *RI* mutants appear to habituate normally when tested in a nonassociative paradigm, habituation of the jump reflex, although RI mutants may spontaneously recover at a faster rate than wild-type. A similar result has been observed for *rutabaga* in another nonassociative paradigm, habituation of a cleaning reflex (Corfas and Dudai, 1989). *Rutabaga* flies were observed to habituate normally with respect to wild-type controls but spontaneous recovery was much faster. I would like to extend the above behavioural analyses of the *RI* mutants, and to include other nonassociative learning paradigms before any significant conclusions can be made about the *RI* mutants affect on nonassociative learning processes. Preliminary evidence (O'Dell and Jamieson, pers. comm.), suggests that homozygous *RI* mutant males behave like *dnc* males in an experience-dependent modification of courtship paradigm (Gailey et al.,

1984; see section 1.3.1). Jamieson and O'Dell (unpublished) observed that *RI* mutant males, like *dnc* males, court females at a frequency significantly higher than wild-type males, even after prior exposure to a fertilised female.

It is clear from the results obtained from various learning and memory paradigms that the effect of the *RI* mutation on acquisition and memory depends critically on the paradigm employed. Nonetheless, it seems to be emerging that the *RI* mutants are disrupted in processes that are common to different learning paradigms. The disruption in associative learning in the *RI* mutants is of particular interest, notably because of the possibility that prolonged activation of protein kinase A (PKA) may represent a molecular correlate of memory (Kandel and Schwartz, 1982; Hawkins et al., 1983; Müller and Spatz, 1989). The implications of the above findings will be discussed in more detail in chapter 6.

Chapter 6

Discussion and future work

6.1 Discussion

The major goal of my Thesis was to develop a reverse genetics approach for the generation of mutants corresponding to any chosen gene and in particular, genes implicated in learning and memory. Chapter 3 describes the development of such a technique, called "site-selected" transposon mutagenesis. This approach allows the isolation of mutations in any cloned gene(s) in any organism in which known transposable elements are active, providing that the mutation rate is not prohibitively low and in which large numbers of independent populations of animals can be easily manipulated (see Chapters 3, 4 and 7).

Using the "site-selected" transposon mutagenesis strategy described above, the regulatory (RI) subunit of a cAMP-dependent protein kinase gene (PKA) was mutated with a view to testing the role of PKA in learning and memory in *Drosophila* (Chapter 4). Two lines were recovered at the RI locus from a P-element insertional mutagenesis, both containing a defective P-element insertion within a ~26 bp region containing multiple transcription start sites which give rise to transcripts encoding the full-length RI gene product. Northern analysis indicates that a transcript of a discrete novel size accumulates in both mutant lines. Both lines are homozygous viable. The success of this reverse genetics strategy is apparent, since a screen based on phenotypic rather than molecular criteria would have been prohibitively laborious. This disruption affects the ability of the flies to learn in a classical olfactory conditioning paradigm (Chapter 5) and supports the involvement of PKA in associative learning of *Drosophila.*

How is the RI mutation producing this effect? Several lines of evidence have implicated cAMP-dependent protein phosphorylation in the learning processes of *Drosophila.* The mutants *dunce* and *rutabaga* are both abnormal in cAMP metabolism and more recently it has been shown that flies deficient for Protein Phosphatase 1 are defective in both nonassociative and associative behaviours (Asztalos et al., 1993). Wild-type flies fed cyclic nucleotide phosphodiesterase inhibitors mimic learning and memory problems associated with these mutants (Folkers and Spatz, 1984; Asztalos et al., 1991)

and transgenic flies expressing a protein inhibitor of PKA were impaired in associative learning (Drain et al., 1991). All these findings implicate cAMP-dependent protein phosphorylation in the neuromodulatory mechanisms underlying *Drosophila* learning and memory. This parallels the physiological findings from studies into learning and memory in the sea snail, *Aplysia californica* (Byrne, 1985; Byrne, 1987; Kandel and Schwartz, 1982; Hawkins et al., 1993). In *Aplysia*, it has been demonstrated that sensitisation of a gill and siphon withdrawal reflex depends on a receptor-activated cAMP cascade. Sensitisation, at the electrophysiological level, is associated with facilitation of synaptic transmission between sensory and motor neurons mediating the reflex. One possible mechanism for facilitating transmission is initiated via the release of a mono-amine neurotransmitter at a "facilitatory" synapse near to the pre-synaptic terminal of a sensory neuron. The neurotransmitter increases cAMP, which acting through the cAMP-dependent protein kinase, inactivates a K^+ channel, thus broadening the action potential which results in prolonged influx of Ca^{2+} and increased neurotransmitter release. Repeated stimulation of the reflex causes a long lasting facilitation of synaptic transmission that requires protein synthesis and may be maintained by the persistent activation of PKA caused by a prolonged decrease in the concentration of regulatory subunits of the kinase (Kandel, 1991). The model was extended by Hawkins et al., (1983) to explain classical conditioning, by conferring on the adenylate cyclase the ability to be activated by Ca^{2+}/Calmodulin (Figure 1.4). They showed that simultaneous activation of adenylate cyclase by the facilitatory transmitter and by free intracellular Ca^{2+} was larger than the sum of the two activation effects applied non-coincidently. Hence, the models of nonassociative and associative learning share aspects of the same secondary messenger cascade.

Protein kinases that are activated by Ca^{2+}/Calmodulin, Ca^{2+}/phospholipid and cAMP are now thought to govern many types of slow (or modulatory) synaptic mechanisms and to mediate many forms of short-term synaptic plasticity (Reichardt and Kelly, 1983; Nairn et al., 1985; Nishizuka, 1986; Schulman, 1988; Griffith et al., 1993). These processes, which do not depend on the synthesis of new proteins and can endure

from minutes to hours, are believed to be the neurophysiological correlates of short-term memory (Kandel and Schwartz, 1982; Schwartz et al., 1983, Goelet et al., 1986). The second messenger molecules operate by maintaining one or more of the kinases in an active state (Hanks et al., 1988). The kinetic features of the synaptic mechanisms, in turn, parallel the kinetic properties of the governing protein kinase because the phosphorylation step appears to determine the time course of the regulatory cascade (Schwartz et al., 1983). For this reason, any molecular mechanism that prolongs the time that the kinase is active would be expected to prolong the duration of the synaptic mechanism, and as a consequence, to prolong the span of the behavioural modification.

With regard to PKA activity, at least three types of post-translational modification, brought about by receptor-mediated second messenger mobilisation or synthesis, may prolong its action: autophosphorylation, alteration of subcellular distribution, and specific proteolysis. In most cases, the post-translational modifications so far described also tend to render the kinase reaction less dependent upon the initial stimulating second messenger. As a consequence, after appropriate previous exposure to the stimulus, the enzyme can continue to phosphorylate protein substrates even when the second messenger falls to the concentration that had been in the naive neuron. These modifications can also produce a primed kinase: prior stimulation can enhance the sensitivity of the enzyme, teaching it to respond lower concentrations of the second messenger (Schwartz and Greenberg, 1987). Persistence and priming both might be considered forms of molecular memory (Crick, 1984; Lisman, 1985). However, none of the modifications of PKA precisely fit the requirements for producing enduring changes. In addition, considerable evidence suggest that new protein synthesis is needed for acquisition of long-term memory (Goelet et al., 1986). The three types of protein modification may instead act in consolidation, bridging the gap between short and long term memory. As yet only two of these modifications have been demonstrated for *Drosophila* PKA; autophosphorylation and proteolysis (Müller and Spatz, 1989).

In *Drosophila*, the genes that encode the catalytic and regulatory subunits have been cloned (Foster, et al., 1984; Foster, et al., 1988; Kalderon and Rubin, 1988) and

characterised. Protein studies (Foster, et al., 1984) have demonstrated that, as in other organisms, there are two forms of *Drosophila* regulatory subunit. A predominant Type II subunit (RII) is found in all tissues and stages, and a Type I subunit (RI) is found at much lower levels in larvae and pupae, but not in embryos or adults. As RI and RII share catalytic subunits, their functional differences are most likely based on subcellular localisation and regulation of the kinase than on differences in substrate specificity. However, some interesting distinctions are apparent. Firstly, RII unlike RI, is subject to autophosphorylation, which modulates its deactivation (Müller and Spatz, 1989). Secondly, from mammalian studies, RII selectively binds to microtubule-associated protein-2 (MAP-2) and a family of A-kinase anchor proteins, all of which are enriched in neuronal tissue and may serve to sequester PKA to specific subcellular sites, presumably co-localising the kinase close to physiological substrates (eg: ion channels)(Bregman et al., 1989; 1991). While RII-anchoring can account for localisation of the PKA holoenzyme, the free catalytic subunit can translocate into specific cellular compartments upon its activation, where it regulates transcription factors such as CREB (Gonzalez and Montminy, 1989). In an elegant series of fluorescent $_{i}$ratio imaging experiments, nuclear translocation of the catalytic subunit has been demonstrated in both mammalian fibroblasts and neurons (Meinkoth et al., 1990; Adams et al., 1991). Thus the regulatory subunits of the PKA holoenzyme may act to sequester the catalytic subunit from the nucleus. Thirdly, PKA can differ in its sensitivity to cAMP so that neuronal responses to basal or increased levels of cAMP may vary in consonance with the isoform composition of PKA in the neuron (Cadd et al., 1990). Fourthly, individual hormones can preferentially activate one or the other holoenzyme form in a tissue specific manner (Scott, 1991) and so distinct pools of PKA can be differentially activated in the same cell in response to different hormones. Finally, Pintér and Friedrich, (1988) showed that the calcium-activated neutral protease, calpain, is found in *Drosophila*, which converts the RII regulatory subunit of PKA by limited proteolysis to a form with less affinity to the catalytic subunit (Müller and Spatz, 1989). This has led to a model were the two signals of conditioning cAMP and Ca^{2+}, converge on PKA (Friedrich, 1990; Aszódi et al, 1991).

It is clear that functional differences between RI and RII forms exist in *Drosophila*, but it may be that both forms are required for normal learning processes, acting either in combination or in isolation. The RI mutation I have isolated is probably a hypomorph and it remains to be seen if the gene is essential (see section 4.5). Since the RII form has not yet been cloned an understanding of the separate roles for the two forms remains to be discovered. From the current incomplete knowledge of the biochemical reactions underlying olfactory learning in *Drosophila*, I assume by analogy with *Aplysia* (Kandel, 1991), that short-term memory resides in the phosphorylation of some protein(s), possibly related to K^+ channels. The learning phenotype observed with the RI mutants is most probably caused by disruption of these physiological events, with ion channel modulation by PKA being a likely candidate (Siegelbaum, 1982; Levitan, 1988; Delgado et al., 1991). It is noteworthy that, like *dunce*, *rutabaga* and *DCO*, RI is expressed at elevated levels in the mushroom body perikarya, as judged by RNA *in situ* hybridisation (see section 4.4). The intriguing aspect of this finding is that three components of the cAMP signalling pathway, cAMP phosphodiesterase, adenylate cyclase and PKA are all expressed in mushroom bodies and produce defects in olfactory learning paradigms. These findings consolidate the involvement of cAMP and PKA in *Drosophila* associative learning as well as the importance of mushroom bodies as centres for learning.

6.2 Future Work

An inherent dilemma in the study of learning mechanisms is that they involve enzymes and other proteins, or their different isoforms, that take part in several cellular processes. Thus the *RI* mutants, apart from the learning anomalies, may have other so far undetected phenotypic traits. It is therefore critical to determine the potential roles of the RI gene product. Is the major requirement a physiological one, rather than a purely developmental requirement? A more detailed neuroanatomical study is required to address some of these problems. In addition, isolation of a null allele (section 4.5) will

allow the introduction of an RI cDNA clone, driven by an inducible promoter, into RI deficient flies. Thus, the importance of RI expression could be determined at different developmental stages. Although this potential pleiotropy does not invalidate the finding that the RI gene is involved in olfactory associative learning, it is also desirable that the mutants be tested in a variety of paradigms employing as many different sensory modalities as possible.

Chapters 4 and 5 have hopefully paved the way for a more detailed study of the molecular genetics of the RI gene and its role in learning and memory. Cloning of the RII gene will help to discover the relative roles of the two forms in relation to *Drosophila* learning processes.

In the final chapter I shall describe the application of site-selected transposon mutagenesis in "tagging" the ductin gene which encodes the 16 kDa proteolipid subunit of the vacuolar H^+-ATPase from *Drosophila melanogaster.* Although of no direct relevance to the study of learning and memory in *Drosophila*, it emphasises the use of this technique in "tagging" any cloned gene.

Chapter 7

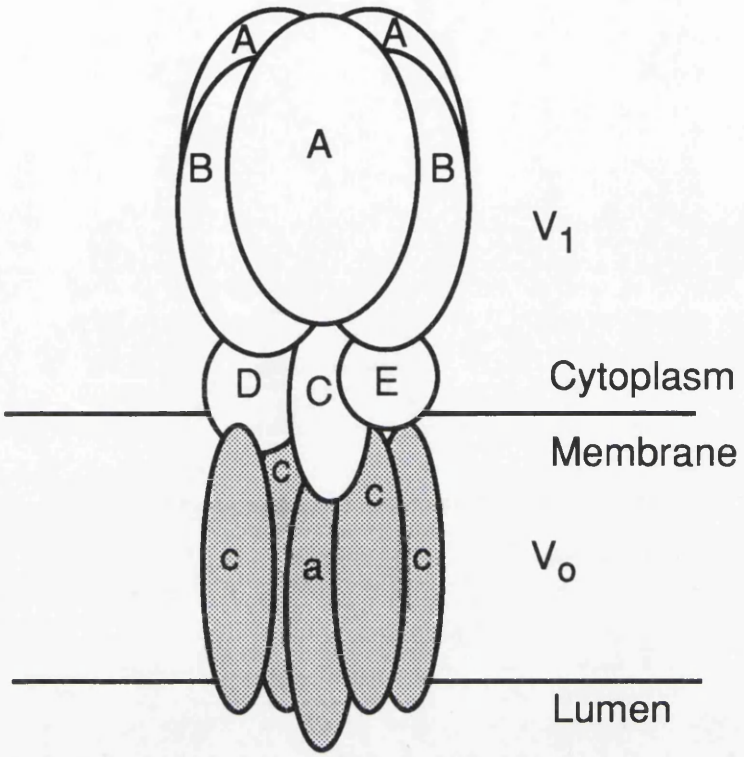
"Site-selected" transposon mutagenesis of the *ductin* gene encoding the 16 kDa proteolipid subunit of the vacuolar H^+-ATPase (V-type ATPase) from *Drosophila melanogaster.*

7.1 Introduction

The vacuolar ATPases (V-type ATPases) are a highly conserved family of proteins responsible for the ATP-dependent transport of ions across the intracellular membranes of eukaryotic cells (Nelson, 1992). They are responsible for acidification of intracellular compartments in eukaryotes, and have recently been found on plasma membranes, where they are thought to play important roles in acid-base regulation or ion channel transport (Gluck, 1992).

The V-type ATPases are multi-subunit protein complexes, related to the F-type ATPases found in mitochondria, chloroplasts, and eubacteria (Nelson, 1989; Nelson, 1992). V-type ATPases are built up of distinct catalytic and membrane sectors (Figure 7.1). The catalytic sector (V_1) is composed of at least five polypeptides denoted as subunits A-E, in order of decreasing molecular mass (Nelson, 1989; Moriyama and Nelson, 1989a; 1989b). The membrane sector (V_o) is comprised of at least two hydrophobic subunits designated a and c (16 kDa proteolipid subunit). The principal polypeptide of the membrane sector is the 16 kDa proteolipid subunit, the transmembrane proton pore is believed to be formed from a hexamer of 16 kDa proteolipid subunits (Nelson, 1992). The 16 kDa subunit is essentially a tandem repeat of two 8 kDa domains and each domain has homology with the 8 kDa subunit c of the F-type ATPase (Nelson and Nelson, 1989). Evolutionary relationships amongst the H^+-ATPases has lead to the theory that F- and V-type ATPases have probably evolved from a common ancestral gene, by a gene duplication event (Mandel et al., 1988; Nelson, 1988; Nelson and Nelson, 1989; Nelson and Taiz, 1989; Gogarten et al., 1989). Although structurally and functionally related, the F- and V-type ATPases are distinguished in eukaryotic cells by the distribution of their genes (Nelson, 1989). V-type ATPase subunits being entirely nuclear encoded, whereas F-type ATPase subunits are encoded both by nuclear and organellar genomes.

In addition, a related, possibly identical 16 kDa proteolipid subunit has also been found to be a component of gap junction-like structures isolated from bovine brain



Subunit	Molecular weight (kDa)
B	57
A	~70
C	44
D	30
E	26
a	20
c	16

Figure 7.1. Schematic model for the minimal subunit structure of V-type ATPases. The model structure is drawn according to the polypeptides present in preparations from fungi, plants and mammals (Nelson, 1989). The stoichiometry of the various subunits in the enzyme is 3A, 3B, 1C, 1D, 1E, 6c and an unknown number of subunit a (Figure adapted from Nelson, 1989).

(Dermietzel et al., 1989). Similar 16 kDa proteins have been reported from other vertebrate and invertebrate sources (Finbow et al., 1983; Finbow et al., 1984; Ryerse, 1989; 1991). It has proved impossible to isolate connexins, the components of vertebrate gap junctions (Warner, 1988), from insect tissues (Berdan and Gilula, 1988). Instead, when gap junction-rich membranes are purified from insect tissue a 16 kDa protein copurifies with the gap junctions (Lane and Finbow, 1988; Ryerse, 1989; 1991; Dow, 1994). Finbow and Pitts, (1993) have controversially suggested that, at least for invertebrates, the actual pore in gap junctions might be formed of the V-type ATPase 16 kDa proteolipid. They have renamed the 16 kDa proteolipid, "ductin" (Holzenburg et al., 1993), and suggest the possibility that the 16 kDa proteolipid has several distinct and more diversified cellular roles, such as in intercellular communications, than had been previously believed.

Meagher et al., (1990), have isolated a 1.1 kb cDNA from *Drosophila* coding for the 16 kDa proteolipid subunit from a larval (3rd instar) cDNA library, as part of a study to understand the structure-function relationships of V-type ATPases in a higher organism. In collaboration with Dr. M.E. Finbow and colleagues at the Beatson Institute of Cancer Research, I carried out a preliminary molecular characterisation of this *ductin* gene encoding the 16 kDa proteolipid subunit of the vacuolar H^+-ATPase, with the ultimate goal to inactivate the locus using the "site-selected" transposon mutagenesis technique, described previously (see chapter 3 and 4).

Genomic organisation of the *ductin* gene was determined by Southern blotting and *in situ* hybridisation to polytene chromosomes. In this way the genomic position and copy number of the *ductin* gene could be determined. A preliminary characterisation of the expression of the *ductin* transcript(s) was determined by Northern blotting experiments. Finally, a "site-selected" transposon mutagenesis was initiated in an attempt to inactivate or "tag" the locus.

7.2 Preliminary molecular characterisation of the *ductin* locus

Drosophila genomic DNA was cleaved with a range of restriction endonucleases, Southern blotted, and probed with the 1.1 kb *ductin* cDNA (Meagher et al., 1990; EMBL accession no. X55979). Hybridisation and washing at high stringency reveals that there is a single gene for this sequence (Figure 7.2a). However, at lower stringencies, other bands are revealed, these suggest the possibility of a multigene family (Figure 7.2b). The *ductin* cDNA was used as a probe to salivary gland polytene chromosome spreads and hybridises uniquely to region 42B1-2 on the right arm of chromosome 2 (Figure 7.2c). This result is consistent with there being a single gene for *ductin.* Northern blot analysis (Figure 7.3) of poly(A)$^+$ mRNA isolated from wild-type adult flies detected a single transcript of approximately 3.0 kb.

7.3 "Site-selected" transposon mutagenesis of the *ductin* locus

"Site-selected" transposon mutagenesis can be applied to any gene, or region of the genome, where sufficient sequence data is available to design suitable primers for PCR amplification, and a probe is available to distinguish *bona fide* amplification products from potential artefactual products. Here I demonstrate this by designing gene-specific primers (see Table 2.2, Materials and Methods) based on partial sequence data from a genomic clone (Figure 7.4) that was previously isolated by hybridisation to the 1.1 kb cDNA corresponding to the *ductin* gene encoding the 16 kDa proteolipid subunit of the V-ATPase (Meagher et al., 1990; Finbow et al., 1994).

Lack of suitable chromosomal deficiencies in the 42B1-2 region necessitated a "site-selected" transposon mutagenesis approach as a way to generate mutants of the *ductin* gene.

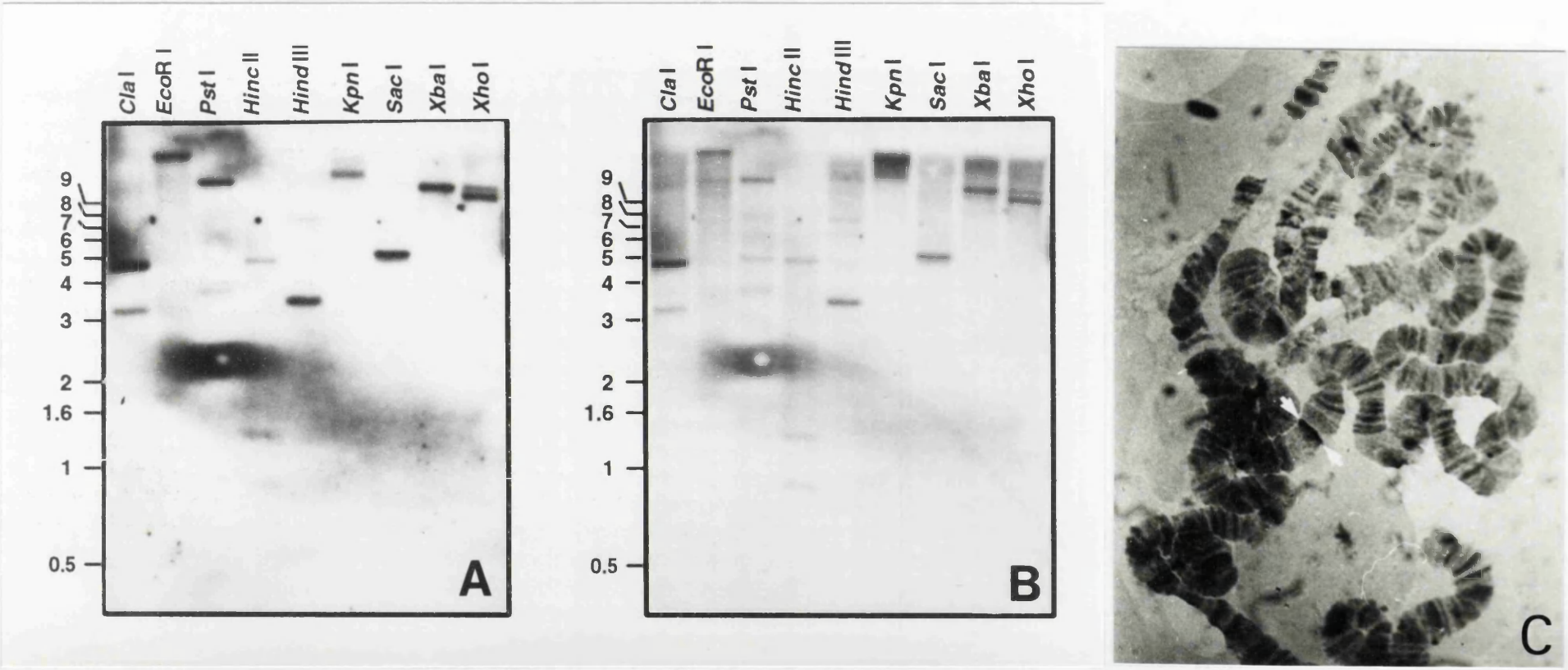


Figure 7.2.

Southern blot of *Drosophila* genomic DNA, probed and washed at high stringency (A) and low stringency (B). Each lane represents 3µg of genomic DNA cleaved with the enzymes shown; none of which cut within the *ductin* cDNA. Aliquots were electrophoresed on a 0.8% agarose gel, blotted to Hybond N , and probed with the *Drosophila* 16 kDa cDNA.. Sizes shown are in kb. (C) Photomicrograph of *in situ* hybridisation to polytene chromosome spread with a biotinylated 1.2 kb *ductin* cDNA. probe. White arrow indicates hybridisation to region 42B1-2 on the right arm of chromosome 2.

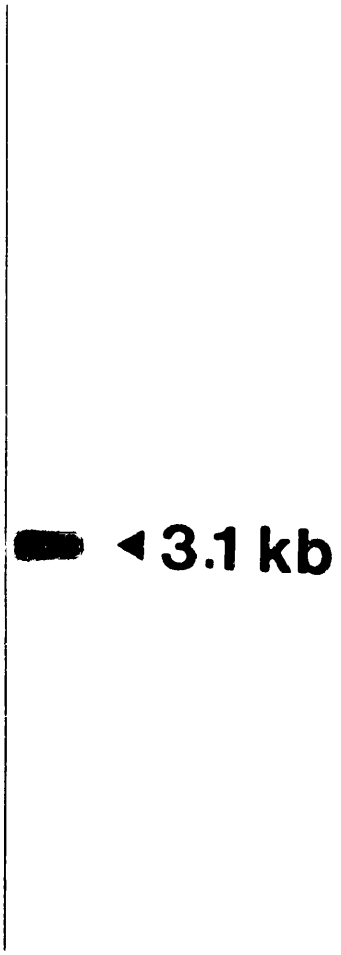


Figure 7.3. RNA blot analysis of *ductin* mRNA. ^{32}P-labeled *ductin* cDNA probe was hybridised to poly(A)$^+$ mRNA prepared from adult flies. The size of the RNA fragment was determined from RNA standards (BRL) and is indicated on the right.

7.3.1 P-element mutagenesis

Random P-element transpositions were generated by a hybrid dysgenic cross as described in section 4.2.1.

7.3.2 "Site-selection"

Although the gene is still under characterisation, primers were designed on available cDNA and genomic sequence information (M. E. Finbow, pers. comm.). The position of the primers are shown in Figure 7.4b. Three gene-specific primers were used with the two P-element primers, P_L and P_R. P-element insertions in the *ductin* gene were detected by PCR amplification with the appropriate primers using egg DNA isolated from mutagenised females. Southern blot analysis of the amplification products using a *ductin* genomic clone was used to confirm *bona fide* amplification products from potential spurious products.

7.3.3 Isolation of P-element Insertions in the *ductin* locus

A screen of 3,000 mutagenised chromosomes (as previously described, section 3.4.2 and 4.2.3) resulted in the identification of six independent P-element insertions 5' to the presumptive initiator codon (Figure 7.4b; 7.5). To ascertain the exact location of these insertions, the PCR products were directly sequenced and the sequence boundary at both ends of the P-element insertions was determined (Figure 7.5).

In a similar manner to *sn*, five of the P-element insertions (*duc2* to *duc6*) have occurred in a small region (~800 bp, 1st intron). These results corroborate the sequence-specific nature of P-element insertions previously observed at *singed* and *white* (O'Hare and Rubin, 1983; Roiha et al., 1988; chapter 3). From Figure 7.5, it can be seen that the P-element in the *ductin* locus of lines *duc5* and *duc6* are inserted at the same nucleotide, but in opposite orientations. Insertions *duc4* and *duc3* differ only by one base in the

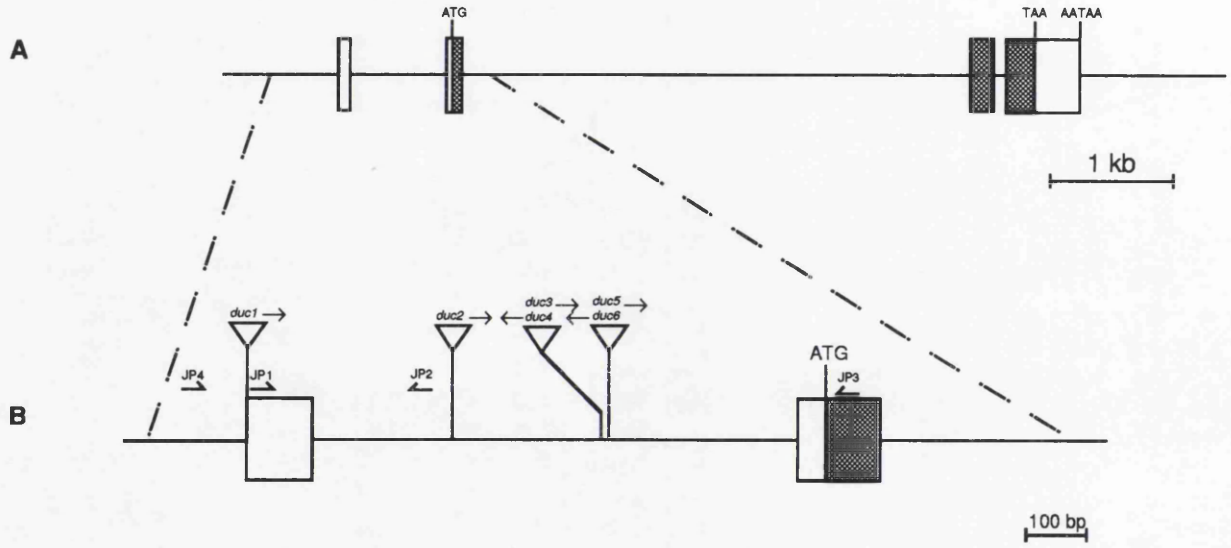


Figure 7.4. Structural organisation of the *ductin* gene.

(A) genomic organisation of the *ductin* locus (Finbow et al., 1994). Map of exons: 4 exons of *ductin* spread over 7 kb were mapped by sequencing all genomic intron-exon junctions, as indicated by boxes. Shaded boxes represent coding sequence.

(B) Sites of P-element insertion and choice of gene-specific primers used. P-element inserts are represented by large triangles above the line, the *arrowhead* corresponds to the right end of the P-element, which is 3' with respect to P-element transcription. Primers are represented by half arrows, to denote direction.

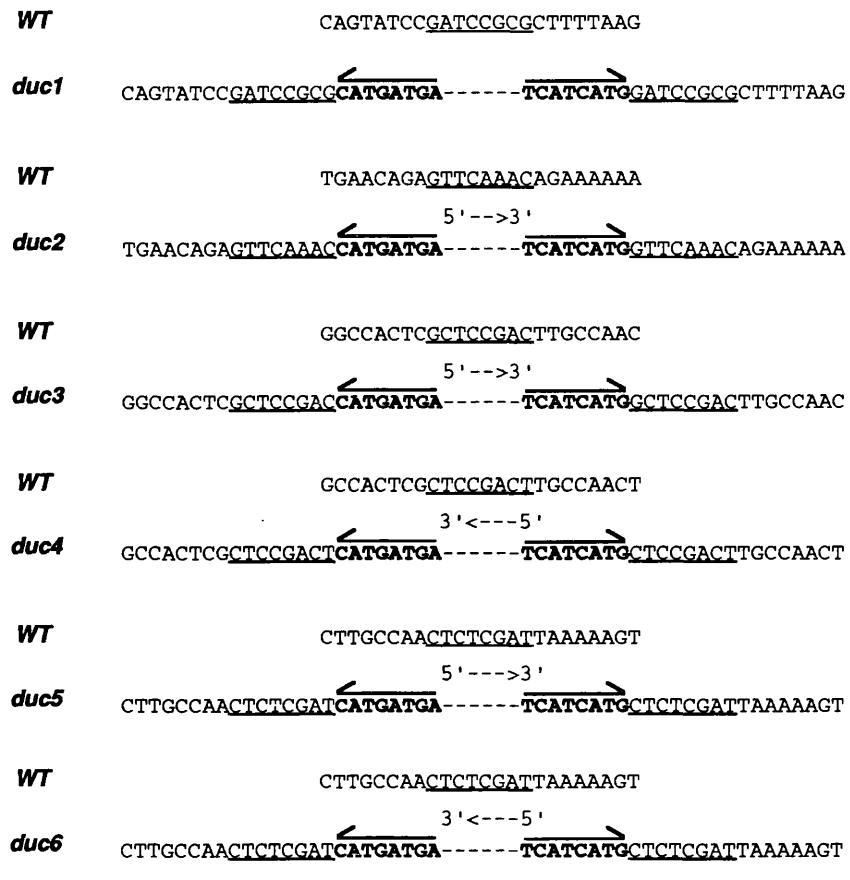


Figure 7.5. DNA sequence changes upon insertion of P-elements in the *ductin* locus. P-element sequences are in bold face, and the 8 bp duplication found at the ends of inserted P-elements is underlined. 5'--->3' corresponds to the direction of P-element transcription. Sequences of the wild-type ductin locus are shown above. The cDNA sequence has been published in the EMBL database (Heidelberg) under accession No. X55979

insertion site. The last insertion, *duc1*, has occurred in the 5' non-coding exon. Thus it would appear that the *ductin* locus is a "hot-spot" for transposition, insertions being recovered at an average frequency of 2 x 10^{-3}. This frequency for P-element insertion at the *ductin* locus is near the upper limit of those observed with similar dysgenic crosses, for example *singed* mutants were recovered at an average frequency of 2.4 x 10^{-3} (Robertson et al., 1988).

To test for possible mutant phenotypes, lines heterozygous for the P-element insertion were crossed with an appropriate second chromosome balancer (*CyO/Sp*). Flies made homozygous for chromosomes carrying insertions *duc1* to *duc6* were viable and fertile, presenting no obvious phenotype.

7.4 Discussion/Future work

Genomic Southern blotting analysis and *in situ* hybridisation to polytene chromosome spreads suggests a single gene for the 16 kDa proteolipid subunit. Similar results have been obtained for another insect species, *Manduca sexta* (Dow et al., 1992). Consistent with their being a single gene, Northern analysis of *Drosophila* adult mRNA reveals a single transcript of ~3.0 kb. It will be of interest to determine whether the same or other mRNA transcripts are present in different developmental stages at similar levels (This work is in progress, L. Dee pers. comm.). Sequence analysis of the previously isolated 1.1 kb cDNA contains a long ORF, encoding a polypeptide of 159 amino acids, that is clearly a 16 kDa subunit. The 1.1 kb cDNA has a 5' UTR of 116 bp, and a 3' UTR of 548 bp, but the length of the mRNA species identified by Northern analysis is much larger, this may reflect more 5' or 3' UTR, further analysis is in progress to address this (L. Dee, pers.comm.).

Using the technique of "site-selected" transposon mutagenesis, six independent P-element insertions were recovered in the *ductin* locus. DNA sequence analysis of the six P-element insertions recovered is consistent with the non random distribution of P-elements seen at other loci (Kidwell, 1987). Intriguingly, five of the P-element insertions

have occurred in a small "hot-spot" region in the first intron of the *ductin* gene, contradicting the theory that probable sites of insertion are 5' regulatory regions (Engels, 1989). However it must be appreciated that most data available for P-element mutagenesis are based on phenotypic rather than molecular criteria. Although some genes will prove to be completely refractile to P-element insertion (Kidwell, 1987; Engels, 1989), molecular screens permit detection of silent insertions, avoiding the bias associated with screens based on phenotypic criteria. Do the insertions in the *ductin* locus disrupt expression of the *ductin* transcript? Northern analysis of poly(A)$^+$ mRNA isolated from flies homozygous for chromosomes carrying insertions *duc*1 to *duc*6 will address this.

One would predict that such a housekeeping gene may be essential, "tagging" of this gene will facilitate the generation of a range of deletions, including nulls, by imprecise excision of the P-element. More recently, as part of the *Drosophila* genome project, a single P-element insertional mutagenesis was carried out for the second chromosome using the P *lacW* transposon (Török et al., 1993). From a screen of ~15,000 insertions on the second chromosome, they isolated ~1700 independent lethal insertion mutants. They estimate that their collection of lethals "comprises all the "hot-spots" and a significant part of the low frequency integration sites available for the P-element on the second chromosome". Since the *ductin* locus is a "hot-spot" for P-element insertions, and maps to the second chromosome, it would seem highly probably that there will be at least one lethal for *ductin* amongst their collection of lethals. Our laboratory is obtaining these lines for other experiments in progress (Y. Guo, pers. comm.), it will be relatively straightforward to screen these lines for P *lacW* insertions in the *ductin* locus by "site-selected" mutagenesis. A marked element will alleviate the need for a molecular approach to generate deletions by imprecise excision.

Hopefully, the experiments presented in this chapter will act as a starting point for the functional analysis of a V-type ATPase in a higher organism.

Appendix

Learning data

Appendix

On the following pages are listed the raw data for the results described in Chapter 5. Abbreviations correspond to the following;

Genotype: CS (Canton-S)
7I5 (*RI*^{7I5})
11D4 (*RI*^{11D4}).

shock: Shocked arm of T-maze.

no shock: Unshocked arm of T-maze.

Mid: Flies trapped in middle point of T-maze.

PI: Performance Index.

PA: Percent Avoidance.

Air: Air only in T-maze arm

MCH : 4-methylcyclohexanol in T-maze arm.

OCT: 3-octanol odour in T-maze arm.
* Denotes shock associated odour.

SR: Spontaneous recovery time

Each cell represents the number of flies for any given experiment (using between 100-150 flies) and the cell on the right hand side, a PI or PA. For the habituation experiments each cell represents a habituation score for an individual fly (the number of trials required to obtain a block of 4 "no-jumps"; section 5.3). Shading of a cell represents a fly that has spontaneously recovered.

Genotype	shock	no shock	Mid	PA
11D4	5	62	13	85.1
11D4	7	111	11	88.1
11D4	17	130	9	76.9
11D4	7	113	8	88.3
11D4	9	98	10	83.2
11D4	10	119	10	84.5
11D4	19	126	11	73.8
11D4	12	107	9	79.8
7I5	8	103	12	85.6
7I5	9	110	13	84.9
7I5	9	88	13	81.4
7I5	3	115	12	94.9
7I5	12	99	12	78.4
7I5	12	119	10	81.7
7I5	13	109	12	78.7
7I5	12	90	9	76.5
CS	4	84	5	90.9
CS	10	89	7	79.8
CS	4	103	8	92.5
CS	15	107	15	75.4
CS	6	65	8	83.1
CS	5	51	9	82.1
CS	9	104	10	84.1
CS	4	78	11	90.2

Table A1.1 Shock Reactivity

Genotype	air	MCH	Mid	PI
11D4	70	9	5	77.2
11D4	79	21	6	58.0
11D4	59	8	6	76.1
11D4	101	9	3	83.6
11D4	70	11	4	72.8
11D4	94	7	4	86.1
11D4	79	18	5	62.9
11D4	74	9	4	78.3
11D4	132	24	5	69.2
11D4	89	18	6	66.4
11D4	87	31	8	47.5
11D4	80	12	3	73.9
11D4	88	24	8	57.1
11D4	86	28	8	50.9
11D4	67	21	4	52.3
11D4	68	7	8	81.3
7I5	102	25	6	60.6
7I5	103	17	5	71.7
7I5	79	14	6	69.9
7I5	99	10	4	81.7
7I5	100	27	5	57.5
7I5	83	15	2	69.4
7I5	70	9	2	77.2
7I5	94	10	2	80.8
7I5	89	24	4	57.5
7I5	70	31	8	38.6
7I5	72	20	5	56.5
7I5	72	13	9	69.4
7I5	81	27	5	50.0
7I5	142	24	6	71.1
7I5	91	23	7	59.6
7I5	80	11	7	75.8
CS	92	18	3	67.3
CS	89	24	4	57.5
CS	105	14	5	76.5
CS	85	9	6	80.9
CS	85	9	2	80.9
CS	111	8	4	86.6
CS	89	15	4	71.2
CS	112	17	3	73.6
CS	85	23	6	57.4
CS	75	11	8	74.4
CS	80	9	5	79.8
CS	55	4	7	86.4
CS	86	17	5	67.0
CS	49	7	5	75.0
CS	70	25	6	47.4
CS	102	31	7	53.4

Table A1.2a Olfactory Acuity: OCT *vs.* AIR.

Genotype	air	OCT	Mid	PI
11D4	100	24	7	61.3
11D4	96	22	5	62.7
11D4	51	6	7	78.9
11D4	100	26	8	58.7
11D4	92	6	3	87.8
11D4	83	9	4	80.4
11D4	97	28	2	55.2
11D4	114	24	3	65.2
11D4	115	15	5	76.9
11D4	83	13	7	72.9
11D4	87	18	4	65.7
11D4	79	19	6	61.2
11D4	98	22	5	63.3
11D4	101	29	8	55.4
11D4	109	29	4	58.0
11D4	121	25	8	65.8
7I5	103	19	3	68.9
7I5	87	43	6	33.8
7I5	119	55	3	36.8
7I5	71	16	7	63.2
7I5	60	9	4	73.9
7I5	81	11	3	76.1
7I5	62	24	4	44.2
7I5	89	17	6	67.9
7I5	105	22	2	65.4
7I5	87	12	5	75.8
7I5	96	15	5	73.0
7I5	95	24	7	59.7
7I5	89	38	3	40.2
7I5	59	11	7	68.6
7I5	74	16	5	64.4
7I5	109	20	5	69.0
CS	117	31	5	58.1
CS	114	18	2	72.7
CS	99	17	7	70.7
CS	127	21	3	71.6
CS	75	18	3	61.3
CS	134	17	5	77.5
CS	70	20	4	55.6
CS	89	9	0	81.6
CS	84	10	3	78.7
CS	77	13	5	71.1
CS	78	11	5	75.3
CS	82	13	6	72.6
CS	85	21	3	60.4
CS	105	18	6	70.7
CS	73	17	4	62.2
CS	83	17	7	66.0

Table A1.2b Olfactory Acuity: MCH *vs.* AIR.

Genotype	Time(min)	OCT*	MCH	Mid1	OCT	MCH*	Mid2	PIo	PIm	PI
CS	0	9	161	6	136	12	5	89.4	83.8	86.6
CS	0	11	126	5	153	8	4	83.9	90.1	87.0
CS	0	4	97	4	109	10	5	92.1	83.2	87.6
CS	0	8	106	3	126	4	6	86.0	93.8	89.9
CS	0	10	123	3	107	8	4	85.0	86.1	85.5
CS	0	4	114	3	102	7	4	93.2	87.2	90.2
CS	15	13	82	5	74	9	2	72.6	78.3	75.5
CS	15	8	75	4	105	25	0	80.7	61.5	71.1
CS	15	20	82	6	97	12	4	60.8	78.0	69.4
CS	15	31	126	5	127	22	5	60.5	70.5	65.5
CS	15	11	56	4	107	17	2	67.2	72.6	69.9
CS	15	13	105	6	135	28	5	78.0	65.6	71.8
CS	30	10	70	6	75	13	4	75.0	70.5	72.7
CS	30	20	75	3	77	9	6	57.9	79.1	68.5
CS	30	13	117	6	89	24	7	80.0	57.5	68.8
CS	30	13	100	6	92	18	6	77.0	67.3	72.1
CS	30	18	67	4	65	13	3	57.6	66.7	62.2
CS	30	18	100	6	76	25	4	69.5	50.5	60.0
CS	60	6	57	5	85	17	3	81.0	66.7	73.8
CS	60	20	114	4	63	14	4	70.1	63.6	66.9
CS	60	21	71	6	62	12	3	54.3	67.6	61.0
CS	60	10	72	3	74	14	5	75.6	68.2	71.9
CS	60	28	63	5	87	25	6	38.5	55.4	46.9
CS	60	13	108	5	96	36	3	78.5	45.5	62.0
CS	180	35	65	6	102	34	5	30.0	50.0	40.0
CS	180	29	50	7	72	23	6	26.6	51.6	39.1
CS	180	32	65	7	47	20	6	34.0	40.3	37.2
CS	180	35	83	3	63	25	8	40.7	43.2	41.9
CS	180	18	56	7	89	24	4	51.4	57.5	54.4
CS	180	20	54	5	89	41	6	45.9	36.9	41.4

Table A1.3 Canton–S retention

Genotype	Time(min)	OCT*	MCH	Mid1	OCT	MCH*	Mid2	PIo	PIm	PI
715	0	13	106	6	54	7	2	78.2	77.0	77.6
715	0	11	67	4	77	8	5	71.8	81.2	76.5
715	0	22	80	6	76	12	2	56.9	72.7	64.8
715	0	25	89	8	97	10	5	56.1	81.3	68.7
715	0	23	79	2	118	23	3	54.9	67.4	61.1
715	0	11	85	0	83	35	2	77.1	40.7	58.9
715	15	20	97	4	86	27	2	65.8	52.2	59.0
715	15	22	74	7	73	19	5	54.2	58.7	56.4
715	15	41	61	5	72	13	8	19.6	69.4	44.5
715	15	35	105	3	102	43	6	50.0	40.7	45.3
715	15	24	90	6	47	21	3	57.9	38.2	48.1
715	15	16	54	5	81	19	5	54.3	62.0	58.1
715	30	23	79	5	75	23	4	54.9	53.1	54.0
715	30	42	104	6	81	29	3	42.5	47.3	44.9
715	30	41	95	5	83	28	7	39.7	49.5	44.6
715	30	24	61	6	86	30	8	43.5	48.3	45.9
715	30	27	76	5	63	22	7	47.6	48.2	47.9
715	30	33	113	6	63	45	6	54.8	16.7	35.7
715	60	27	80	4	53	24	3	49.5	37.7	43.6
715	60	25	72	4	56	22	5	48.5	43.6	46.0
715	60	20	48	8	54	37	7	41.2	18.7	29.9
715	60	27	49	9	99	44	9	28.9	38.5	33.7
715	60	41	58	5	43	32	7	17.2	14.7	15.9
715	60	29	91	5	89	38	7	51.7	40.2	45.9
715	180	36	50	4	40	36	3	16.3	5.3	10.8
715	180	32	54	5	70	29	4	25.6	41.4	33.5
715	180	38	49	0	66	51	4	12.6	12.8	12.7
715	180	28	72	5	63	35	3	44.0	28.6	36.3
715	180	33	78	3	57	23	5	40.5	42.5	41.5
715	180	32	84	2	42	30	5	44.8	16.7	30.7

Table A1.3 RI^{715} retention

Genotype	Time(min)	OCT*	MCH	Mid1	OCT	MCH*	Mid2	PIo	PIm	PI
11D4	0	20	64	4	116	18	3	52.4	73.1	62.8
11D4	0	17	59	6	107	11	5	55.3	81.4	68.3
11D4	0	21	68	8	89	15	3	52.8	71.2	62.0
11D4	0	29	101	7	126	13	5	55.4	81.3	68.3
11D4	0	9	108	7	110	25	6	84.6	63.0	73.8
11D4	0	17	124	4	93	34	5	75.9	46.5	61.2
11D4	15	31	102	6	84	17	3	53.4	66.3	59.9
11D4	15	8	58	3	71	15	3	75.8	65.1	70.4
11D4	15	30	65	6	93	23	8	36.8	60.3	48.6
11D4	15	31	91	7	89	19	5	49.2	64.8	57.0
11D4	15	19	94	6	82	23	5	66.4	56.2	61.3
11D4	15	28	96	8	69	29	4	54.8	40.8	47.8
11D4	30	50	71	6	59	14	3	17.4	61.6	39.5
11D4	30	35	87	3	102	21	6	42.6	65.9	54.2
11D4	30	33	70	5	86	21	7	35.9	60.7	48.3
11D4	30	37	89	7	88	21	5	41.3	61.5	51.4
11D4	30	30	70	7	73	26	6	40.0	47.5	43.7
11D4	30	15	80	5	101	41	7	68.4	42.3	55.3
11D4	60	40	93	5	71	28	4	39.8	43.4	41.6
11D4	60	43	78	3	75	29	4	28.9	44.2	36.6
11D4	60	37	46	8	113	37	9	10.8	50.7	30.8
11D4	60	42	59	6	108	25	8	16.8	62.4	39.6
11D4	60	35	56	3	59	21	4	23.1	47.5	35.3
11D4	60	32	95	6	54	30	7	49.6	28.6	39.1
11D4	180	42	52	5	66	32	7	10.6	34.7	22.7
11D4	180	30	43	5	63	39	3	17.8	23.5	20.7
11D4	180	60	69	9	65	21	8	7.0	51.2	29.1
11D4	180	44	66	6	108	32	3	20.0	54.3	37.1
11D4	180	33	61	6	70	29	5	29.8	41.4	35.6
11D4	180	27	88	4	57	31	2	53.0	29.5	41.3

Table A1.3 *RI* ^{11D4} retention

Experiment 1 SR = 120 sec	
Genotype	Habituation score
CS	8
CS	3
CS	7
CS	6
CS	13
CS	8
CS	9
CS	12
CS	14
CS	3
CS	2
CS	5
CS	2
CS	5
CS	6
CS	8
7I5	12
7I5	15
7I5	1
7I5	2
7I5	5
7I5	6
7I5	7
7I5	8
7I5	9
7I5	2
7I5	5
7I5	1
7I5	5
7I5	6
7I5	5
7I5	14
11D4	4
11D4	7
11D4	12
11D4	4
11D4	1
11D4	1
11D4	3
11D4	2
11D4	9
11D4	14
11D4	12
11D4	4
11D4	7
11D4	5
11D4	3
11D4	9

Experiment 2 SR = 300 sec	
Genotype	Habituation score
CS	7
CS	2
CS	4
CS	13
CS	8
CS	11
CS	2
CS	10
CS	13
CS	14
CS	8
CS	6
CS	6
CS	7
CS	4
CS	11
7I5	13
7I5	10
7I5	6
7I5	7
7I5	8
7I5	10
7I5	2
7I5	1
7I5	6
7I5	5
7I5	7
7I5	10
7I5	17
7I5	4
7I5	3
7I5	8
11D4	2
11D4	7
11D4	5
11D4	10
11D4	11
11D4	12
11D4	2
11D4	5
11D4	11
11D4	6
11D4	5
11D4	7
11D4	8
11D4	10
11D4	11
11D4	9

Experiment 3 SR = 900 sec	
Genotype	Habituation score
CS	5
CS	1
CS	16
CS	3
CS	7
CS	5
CS	12
CS	7
CS	5
CS	3
CS	1
CS	3
CS	2
CS	9
CS	14
CS	8
7I5	8
7I5	3
7I5	12
7I5	11
7I5	2
7I5	10
7I5	18
7I5	6
7I5	6
7I5	2
7I5	2
7I5	13
7I5	5
7I5	14
7I5	13
7I5	3
11D4	2
11D4	15
11D4	4
11D4	1
11D4	8
11D4	7
11D4	15
11D4	12
11D4	3
11D4	9
11D4	7
11D4	7
11D4	13
11D4	10
11D4	10
11D4	7

Table A1.4 Habituation Scores

Experiment 4 SR = 1200 sec	
Genotype	Habituation score
CS	7
CS	6
CS	15
CS	14
CS	9
CS	3
CS	1
CS	9
CS	15
CS	13
CS	2
CS	5
CS	7
CS	8
CS	9
CS	11
7I5	3
7I5	4
7I5	16
7I5	8
7I5	7
7I5	13
7I5	12
7I5	9
7I5	7
7I5	8
7I5	10
7I5	2
7I5	7
7I5	4
7I5	11
7I5	7
11D4	10
11D4	7
11D4	8
11D4	7
11D4	13
11D4	15
11D4	10
11D4	7
11D4	15
11D4	1
11D4	3
11D4	11
11D4	7
11D4	8
11D4	9
11D4	7

Experiment 5 SR = 1800 sec	
Genotype	Habituation score
CS	6
CS	2
CS	14
CS	7
CS	3
CS	2
CS	10
CS	5
CS	3
CS	1
CS	14
CS	8
CS	4
CS	3
CS	3
CS	7
7I5	7
7I5	1
7I5	10
7I5	8
7I5	8
7I5	6
7I5	5
7I5	3
7I5	8
7I5	3
7I5	2
7I5	7
7I5	10
7I5	6
7I5	7
7I5	8
11D4	14
11D4	7
11D4	8
11D4	3
11D4	4
11D4	8
11D4	10
11D4	7
11D4	3
11D4	4
11D4	3
11D4	6
11D4	7
11D4	4
11D4	2
11D4	1

Table A1.4 Habituation Scores (cont..)

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